ACTA PHYSIOLOGICA SCANDINAVICA

ACTA PHYSIOL. SCAND

VOL. 80

Vol. 80. INDEX

Fasc 1. (September 1970)

Thermogenesis of Brown Adipose Tissue Reflected in Mitochondrial Respiratory Control By H T Andersen, E N Christiansen H J Gray and J I Pedersen 1

Variation in Total Body Water with Muscle Glycogen Changes in Man By K E Olsson and B Saltin 11

Blood Flow and Oxygen Extraction in the Cat Uvea at Normal and High Intraocular Pressures By A Alm and A Bill 19

Effects of Feeding States on Lipid Radioactivity in Liver, Muscle and Adipose Tissue after Injection of Labelled Glucose in the Rat By P Björntorp, M Krotkiewski B Larsson and Z Somlo-Szücs 29

Determination of Cortical Blood Flow in Rabbit Femur by Radioactive Microspheres By P K M Lunde and K Michelsen 39

The Incorporation of Cholesterol into the Nervous System of the Newly Hatched Chick By O Svanberg 45

Plasma Skimming in the Intestinal Tract By M Jodal and O Lundgren 50

Blood Flow through Limb Muscles during Heavy Rhythmic Exercise By B Folkow, P Gaskell and B A Waaler 61

Distribution of Choline Acetyltransferase in the Hearts of Mammals By J Ekström 73

Time Relations of Degeneration Mydriasis and Degeneration Vasoconstriction in the Rabbit Ear after Sympathetic Denervation Effect of Bretylium By E H Bárány and G Treister 79

Background of Increased Flow Resistance and Vascular Reactivity in Spontaneously Hypertensive Rats By B Folkow M Hallbäck Y Lundgren and L Weiss 93

The Degeneration Contraction of a Sympathetically Innervated Smooth Muscle in the Rat after Reserpine Inhibition of Monoamine Oxidase or Tyrosine Hydroxylase By D Lundberg 107

The Effect of Dinitrophenol on Secretory Potentials Secretion and Potassium Accumulation in the Perfused Cat Submandibular Gland By O H Petersen 117

Exchange of Glucose between Plasma Brain Extracellular Fluid and Cerebral Ventricles in Cats and Effects of Intraventricular Acetazolamide and Insulin By H E Brondstedt 122

Adrenergic Excitatory Influences on Initiation and Conduction of Electrical Activity in the Rat Portal Vein By B Ljung and L Stage 131

Short Communication

The Effect of Anesthetics on the Tissue Lactate Pyruvate Phosphocreatine ATP and AMP Concentrations and on Intracellular pH in the Rat Brain By L Nilsson and B K Siesjö 142

Fasc 2 (October 1970)

Effect of Hypotonic Mannitol and Saline Load on Diluting Capacity in Man By A Aperia O Broberger and H Feychting 145

Mechanisms Involved in the Cat's Blink Reflex By Chr Lindquist and A Mårtensson 149

Renal Reabsorption and Metabolic Conversion of Galactose in the Cat By S N Rasmussen 160

Potentiation by Various Smooth Muscle Stimulants of an Isolated Sympathetic Nerve Seminal Vesicle Preparation from the Guinea Pig By N O Sjöstrand and G Swedin 172

Single Unit Analysis of Mechanoreceptor Activity from the Human Glabrous Skin By M Knibestöl and Å B Vallbo 178

Projections to the Cerebral Cortex from Afferents of the Interosseous Nerves of the Cat By H Silfvenius 196

Quantitative Correlation between Degranulation and Histamine Release Following Exposure of Rat Mast Cells to Compound 48/80 *in vitro* By R. Nosál, S. A. Slorach and B. Uvnäs 215
Effects of Increased Liver Metabolism of Nicotine on Its Uptake, Elimination and Toxicity in Mice By T. Stålhandske 222
The Effect of Anesthetics upon Labile Phosphates and upon Extra- and Intracellular Lactate, Pyruvate and Bicarbonate Concentrations in the Rat Brain By L. Nilsson and B. K. Siesjö 235
Acetylation of p-Aminohippuric Acid in the Kidney. Renal Clearance of p-Aminohippuric Acid and N[4] Acetylated p-Aminohippuric Acid in Pigs By N. Gyrd-Hansen and F. Rasmussen 249
The Circadian Fluctuations of the Amount of Free Phosphate and of the Activity of Acid Phosphatase in the Kidneys of Mice and the Effect of UV Radiation upon this Rhythm By P. E. Lindahl and J. Surowiak 254
Antagonism by Calcium of the Inhibitory Action of Prostaglandin E_2 on Sympathetic Neurotransmission in the Cat Spleen By P. Hedqvist 269
Ontogenesis of Peripheral Adrenergic Neurons in the Rat. Pre- and Postnatal Observations. By J. de Champlain, T. Malmfors, L. Olson and Ch. Sachs 276

Fasc. 3 (November 1970)

Effect of Prostaglandin E_1 on Fat Mobilizing Lipolysis in Rat Adipose Tissue in Relation to the Nutritional Condition By L. A. Carlson and H. Micheli 289
The Relationship between Arylesterase Activity and Growth in Rats By K. B. Augustinsson and B. Henricson 295
The Effect of Glucose on Histamine Release from Isolated Rat Peritoneal Mast Cells Induced by Adenosine 5' Triphosphate By B. Diamant and C. Peterson 299
Synthesis of Noradrenaline from 3,4-Dihydroxyphenylalanine (DOPA) and Dopamine in Adrenergic Nerves of Mouse Atrium — Effect of Reserpine, Monoamine Oxidase and Tyrosine Hydroxylase Inhibition By G. Jonsson and Ch. Sachs 307
Effects of Some Adrenergic Neuron Blockers, Related Quaternary Ammonium Compounds and Guanidine Derivatives on Degenerating Adrenergic Nerves in the Conscious Rat By D. Lundberg 323
Cytoplasmic Distribution of Endogenous and Exogenous 5-Hydroxytryptamine in Rat Peritoneal Mast Cells By S. E. Jansson 345
[illegible] 353
[illegible] study By J. Nickels 360
Correlation between the Rise Time of Miniature End Plate Potential and the Distance of the Recording Microelectrode from the Myoneural Junction By J. Nickels 370
Contraction Times of Twitches Evoked by H Reflexes By F. Buchthal and H. Schmalbruch 378
Circulatory Effects of Interruption and Stimulation of Cardiac Vagal Afferents. By B. Öberg and S. White 383
The Role of Vagal Cardiac Nerves and Arterial Baroreceptors in the Circulatory Adjustments to Hemorrhage in the Cat By B. Öberg and S. White 395
[illegible] 404
In[illegible] Erythron By E. [illegible]

Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish I The Existence of a Double Inhibitory Feedback By J K S JANSEN A NJÅ and L WALLOE 420

The Significance of Age for the Phosphatase Activity of Seminal Fluid By C. WIKSTROM 426

Short Communications

Preferential Secretion of Newly Formed Noradrenaline in the Perfused Rabbit Heart By L. STJÄRNE and Å WENNMALM 428

Colchicine Induced Delay of the Degeneration Release of Sympathetic Transmitter in the Conscious Rat By D LUNDBERG 430

Fasc 4 (December 1970)

Metabolism of Subcutaneously Injected ^{14}C-Histamine in *Myxine Glutinosa* By B HOLSTEIN 433

The Response of RNA Synthesis to Mitotic Regulation in the Erythron By E KIVILAAKSO 436

Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish II Reflex Input Segmental Distribution and Output Relations By J K S JANSEN A NJÅ and L WALLOE 443

Effects of Prostaglandin E_1 in Canine Subcutaneous Adipose Tissue *in Situ* By B B FREDHOLM and S ROSELL 450

Reduction in Pulmonary Blood Volume after Blood Loss By P AARSETH 459

A Double Isotope Method for Determination of the Miscible Inorganic Sulfate Pool of the Mouse Applied to *in vivo* Studies of Sulfate Incorporation into Costal Cartilage By G HERBAI 470

Synchronization of Activity in Afferent Nerve Branches within the Frog's Muscle Spindle By D OTTOSON and G M SHEPHERD 492

Regional Differences in the Incorporation Rates of ^{3}H Acetate and ^{35}S Sulfate into Chondroitin Sulfate of Mouse Costal Cartilage *in Vitro* By G HERBAI and L LINDAHL 502

Potentiating Action of Desmethylimipramine on the Cardiovascular Effects of Noradrenaline and Some Other Pressor Agents By A ÅSTROM 510

Studies on the Mechanism of Action of Lithium Ions I The Effect of Lithium Ions on the Impulse Activity of the Crayfish Stretch Receptor Neuron By E GIACOBINI and M STEPITA KLAUCO 519

Studies on the Mechanism of Action of Lithium Ions II Potassium Sensitive Influx of Lithium Ions into the Crayfish Stretch Receptor Neuron Determined by Microflamephotometry By E GIACOBINI S HOVMARK and M STEPITA KLAUCO 528

Regional Distribution of Red Cells Plasma and Blood Volume in the Intestinal Wall of the Cat By M JODAL and O LUNDGREN 533

Ventilatory Responses to Hypoxia and Hypercapnia with External Airway Resistance By J B BARNETT and B RASMUSSEN 538

Discharge Patterns in Human Muscle Spindle Afferents during Isometric Voluntary Contractions By Å B VALLBO 552

Metabolic Effects of Prolonged Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue By B B FREDHOLM and J KARLSSON 567

Abstracts from Meeting of the Scandinavian Physiological Society in Stockholm 20—21 November 1970

Demonstrations

Microelectrode Recording from the Facial Nucleus in the Cat By C. LINDQUIST 1A

On the Dynamic Properties of the Middle Ear Reflexes By E. BORG 2A

Vasopressin Content in the Neurohypophysis of Rats during Overhydration and Rehydration By N A THORN H VILHARDT E ENGBERG I KJELDSEN and K LARSEN 3A
Studies on Mammary Respiratory Metabolism and Mammary Development after Thyroidectomy in the Rabbit By A NILSSON T NILSSON and A NORGREN 4A
Studies on the Minimal Exposure Time for Androgen to Evoke Sterility in Female Rats. By T ALKLINT and A NORGREN 5A
Inhibition by Prostaglandin E_1 of Vascular Response to Sympathetic Nerve Stimulation *in Vitro* By P HEDQVIST 6A
Biochemistry of Cathecholamine Storage A Comparison between Sympathetic Nerve Trunk and Adrenomedullary Vesicles By K B HELLE H LAGERCRANTZ and L. STJARNE 7A
Reflex Relaxation of the Stomach Elicited from Heart Receptors By H ABRAHAMSSON and P THORÉN 8A
Renal Vascular Resistance in Spontaneously Hypertensive Rats By B FOLKOW M HALLBACK Y LUNDGREN and L. WEISS 9A
The Projection of Group I Muscle Afferents from the Hindlimb to the Contralateral Thalamus of the Cat By S LANDGREN and H SILFVENIUS 10A
A Projection from the Nucleus Z to the Ventral Nuclear Complex of the Thalamus in the Cat By J BOIVIE G GRANT and H SILFVENIUS 11A
Recurrent Depression from Motor Axon Collaterals of Supraspinal Inhibitory Effects in Motoneurones By H HULTBORN and M UDO 12A
Depression of Ia IPSPs in Spinal Border Cells by Impulses in Recurrent Motor Axon Collaterals By B GUSTAFSSON and S LINDSTROM 13A
Visually Guided Behaviour after Total Neonatal Removal of the Neocortex in the Cat By K NORRSELL and U NORRSELL 14A
An Aquatic Swim Mill By P-O ÅSTRAND and S ENGLESSON 15A
The Stimulating and Inhibitory Effect of Carbamoylcholine on Decamethonium Uptake in Mouse Kidney Slices By J HOLM 15A
Effect of Training with Eccentric Muscle Contractions on Human Skeletal Muscle Metabolites By F B PETERSEN and H G KNUTTGEN 16A
Energy Metabolism during Isometric Exercise at Different Temperatures of M Quadriceps Femoris in Man By R H T EDWARDS R C. HARRIS E HULTMAN L. KAIJSER D KOH and L. O NORDESJO 17A

Communications

Ventilatory Response to CO_2 in Hyperbaric Environments By L. FAGRAEUS and C M HESSER 19A
Regulation of Depth and Rate of Breathing in Cat and Man By F J CLARK and C. von EULER 20A
High Frequency Low Tidal Volume Positive Pressure Ventilation By A JONZON P A ÖBERG G SEDIN and U SJOSTRAND 21A
Increased Activity in Vagal Cardiac Afferents Correlated to the Appearance of Reflex Bradycardia during Severe Hemorrhage in Cats By B ÖBERG and P THORÉN 22A
Nervous Influence on the Pulmonary Blood Volume By P AARSETH 23A
Microneurographic Analysis of the Fibre Spectrum of Human Sensory Nerve Fascicles By R G HALLIN and H E TOREBJORK 24A
Excitatory and Inhibitory Propriospinal Pathways from Lumbo Sacral to Cervical Segments in the Cat By S MILLER 25A
Ethanol Electrical Stimulation and Net Movements of Sodium and Potassium in Rat Brain Tissue *in Vitro* By P NIKANDER and H WALLGREN
Proportionality between g Strophantin Binding Capacity and ATPase Activity B HANSEN

Studies on the Action of Various Cyclic Nucleotides and Their Metabolites on the Synthesis of Corticosterone *in Vitro* By P BIECK H VAPAATALO and E WESTERMANN 28A
Serum Non Esterified Fatty Acids and Plasma Glycerol as Indicators of Fat Mobilization in Pregnant Sheep Subjected to Cold Stress By N ASTRUP, A AULIE, J F NEDKVITNE and W VELLE 30A
Dynamics of Fluid Transfer between the Intra and Extravascular Compartments during Exercise By J LUNDVALL, S MELLANDER H WESTLING and T WHITE 31A
Increased Forearm Blood Flow during Ketoacidosis in Man By S L NIELSEN 32A
Physiological Interrelations during Intermittent and Continuous Exercise with the Same Average Power Output in Man By R H T EDWARDS C M HESSER, I JACK A MELCHER, A M McNINCH R D W WELLS and O WIGERTZ 33A
Hyperpolarizing Actions of Noradrenaline in Spinal Motoneurones By I ENGBERG and A THALLER 34A
Is Interruption of the Nigro-Striatal Dopamine System Producing the 'Lateral Hypothalamus Syndrome'? By U UNGERSTEDT 35A
Autonomic Innervation of the Smooth Muscle Sphincter in the Swimbladder of a Fish (*Gadus morrhua*) By S NILSSON 36A
Differences in Effects of α Methyltyrosin on Various Peripheral Noradrenaline Stores of the Rat By G SWEDIN 37A
Interference of Membrane Pump Blocking and α Receptor Blocking Drugs with Noradrenaline Release from Isolated Tissues By L O FARNEBO and B HAMBERGER 38A
Studies on Ribonucleic Acids of the Mammary Gland of Pregnant and Lactating Rabbits By A DEUTSCH and A NORGREN 39A
Renovascular Hypertension in Rats Immunized with Angiotensin By I EIDE 40A
The Effect of Slight Hypothermia on Hepatic Excretory Function in Cats By N KRARUP 41A
Changes in Spinal Excitability during an Auditory Vertex Response in Man By H FRUHSTORFER T JARVILEHTO J LUMIO and P SOVERI 42A
Response Patterns of Human Muscle Spindle Endings to Isometric Voluntary Contractions By A B VALLBO 43A
lamo-Cortical Rhythmicity during Reversible Visual Deafferentation By S ANDERSSON E HOLMGREN and T IVARSSON 44A
nfluence of Plasma Hypertonicity on Blood Viscosity Studied *in Vitro* and in an Isolated Vascular Bed By S N RASMUSSEN 45A
Efflux of Prostaglandins in Lymph from Scalded Tissues By E ÄNGGÅRD G ARTURSON and C E JONSSON 46A
The Permeability of the Blood Brain Barrier to Lactate and Pyruvate By C CRONE and S C SORENSEN 47A
Local Regulation of Vascular Cross Section during Changes in Femoral Arterial Blood Flow in Dogs By F KIIL M LIE and O M SEJERSTED 48A

Supplements

Supplementum 347 Effect of Posture on Circulation and Respiration at Rest and during Exercise in Heart Disease By H ÅSTROM
Supplementum 348 Growth Characteristics of Adrenergic Nerves in the Adult Rat By L OLSON and T MALMFORS
Supplementum 349 Prejunctional Supersensitivity in the Rat Portal Vein as Related to Its Pattern of Innervation By B JOHANSSON B LJUNG T MALMFORS and L OLSSON
Correlation between Noradrenaline Release and Effector Response to Nerve Stimulation in Rat Portal Vein *in vitro* By J HAGGENDAL B JOHANSSON J JONASON and B LJUNG

Nervous and Myogenic Mechanisms in the Control of a Vascular Neuro-effector System. By B. Ljung

Supplementum 350. On the Relation between Blood Pressure and Blood Flow in the Canine Brain with Particular Regard to the Mechanism Responsible for Cerebral Blood Flow Autoregulation. By B. Ekstrom Jodal

Supplementum 351. Factors Influencing the Autonomic Component of the Defence Reaction. By B. Lisander

Supplementum 352. Postnatal Development of Spinal Anterior Horn Neurones in Normal and Undernourished Rats. A Quantitative Cytochemical Study. By M. Haltia.

Supplementum 353. Contractility of the Isolated Human Fetal Heart. Influence of contraction rate, acid base parameters and a local anaesthetics. By G. Gennser, K. E. Andersson and E. Nilsson

Supplementum 354. Studies on the Sympathetic Regulation of Circulation and Metabolism in Isolated Canine Subcutaneous Adipose Tissue. By B. B. Fredholm

INDEX AUCTORUM

AARSETH P Pulmonary Blood Volume 459
ALM A and A BILL Oxygen Extraction in Uvea 19
ANDERSEN H T E N CHRISTIANSEN H J GRAV and J I PEDERSEN Brown Fat Thermogenesis 1
APERIA A O BROBERGER and H FEYCHTING *Hypotonic Mannitol and Saline Load* 145
ÅSTRÖM A Potentiating Action of DMI 510
AUGUSTINSSON K B and B HENRICSON Arylesterase Activity and Growth 295
BÁRÁNY E H and G TREISTER Degeneration Mydriasis and Vasoconstriction 79
BARNETT T B and B RASMUSSEN Ventilatory Responses with Airway Resistance 538
BILL A and A ALM Oxygen Extraction in Uvea 19
BJORNTORP P M KROTKIEWSKI B LARSSON and Z SOMLO SZÜCS Lipogenesis *in Vivo* 29
BROBERGER O A APERIA and H FEYCHTING Hypotonic Mannitol and Saline Load 145
BRONDSTED H E Brain Glucose Acetazolamide and Insulin 122
BUCHTHAL F and H SCHMALBRUCH Contraction Time of Twitches 378
CARLSON L A and H MICHELI PGE_1 on Fat Mobilizing Lipolysis 289
de CHAMPLAIN J T MALMFORS L OLSON and CH SACHS Ontogenesis of Adrenergic Neurons 276
CHRISTIANSEN E N H T ANDERSEN H J GRAV and J I PEDERSEN Brown Fat Thermogenesis 1
DIAMANT B and C PETERSON ATP Induced Histamine Release 295
EKSTROM J Choline Acetyltransferase in the Heart 73
FEYCHTING H A APERIA and O BROBERGER Hypotonic Mannitol and Saline Load 145
FOLKOW B P GASKELL and B A WAALER Muscle Blood Flow during Heavy Exercise 61
FOLKOW B M HALLBACK Y LUNDGREN and L WEISS Flow Resistance in Spontaneously Hypertensive Rats 93
FREDHOLM B B and J KARLSSON *Stimulated Fat Metabolism* 567
FREDHOLM B B and S ROSELL Noradrenaline and Adipose Tissue 404
FREDHOLM B B and S ROSELL PGE_1 in Adipose Tissue 450
GASKELL P B FOLKOW and B A WAALER Muscle Blood Flow during Heavy Exercise 61
GIACOBINI E and M STEPITA KLAUCO Lithium and Stretch Receptor Neuron I 519
GIACOBINI E S HOVMARK and M STEPITA KLAUCO Lithium and Stretch Receptor Neuron II 578
GRAV H J H T ANDERSEN E N CHRISTIANSEN and J I PEDERSEN Brown Fat Thermogenesis 1
GYRD-HANSEN N and F RASMUSSEN PAH Clearance in Pigs 249
HALLBACK M B FOLKOW Y LUNDGREN and L WEISS Flow Resistance in Spontaneously Hypertensive Rats 93
HEDQVIST P Prostaglandin Antagonism by Calcium 269
HENRICSON B and K B AUGUSTINSSON Arylesterase Activity and Growth 295
HERBAI G Sulfate Uptake into Cartilage 470
HERBAI G and U LINDAHL Biosynthesis of Chondroitin Sulfate 50?
HOLSTEIN B Histamine Metabolism in *Myxine* 433
HOVMARK S E GIACOBINI and M STEPITA KLAUCO Lithium and Stretch Receptor Neuron II 578
JANSEN J K S A NJA and L WALLOE Inhibitory Control of Stretch Receptors I 470
JANSEN J K S A NJA and L WALLOE Inhibitory Control of Stretch Receptors II 483
JANSSON S E *5-HT in Mast Cells* 315
JODAL M and O LUNDGREN Intestinal Plasma Skimming 50
JODAL M and O LUNDGREN Intestinal Blood Volume Distribution 533

JONSSON G and CH SACHS Noradrenaline Synthesis in Mouse Atrium 307
KARLSSON J and B B FREDHOLM Stimulated Fat Metabolism 567
KEWENTER J P E PAHLIN and B STORM Nerve Stimulation on Jejunal Motility 353
KIVILAAKSO E Mitotic Stimulator in Hypoxic Serum 412
KIVILAAKSO E RNA Synthesis and Mitotic Regulation 436
KNIBESTOL M and A B VALLBO Mechanoreceptors in Human Glabrous Skin 178
KROTKIEWSKI M P BJORNTORP B LARSSON and Z SOMLO-SZÜCS Lipogenesis *in Vivo* 29
LARSSON B P BJORNTORP M KROTKIEWSKI and Z SOMLO-SZÜCS Lipogenesis *in Vivo* 29
LINDAHL P E and J SUROWIAK *Circadian Fluctuations of Acid Phosphatase* 254
LINDAHL, U and G HERBAI Biosynthesis of Chondroitin Sulfate 502
LINDQUIST CHR and A MÅRTENSSON Blink Reflex 149
LJUNG B and L STAGE NA Control of Vascular Automaticity 131
LUNDBERG D Monoamine Oxidase Inhibitors and Denervation 107
LUNDBERG D Neuron Blockers and Degeneration Contraction 323
LUNDBERG D Colchicine Induced Delay of the Degeneration 430
LUNDE P K M and K MICHELSEN Cortical Blood Flow in Rabbit Femur 39
LUNDGREN O and M JODAL, Intestinal Plasma Skimming 50
LUNDGREN O and M JODAL Intestinal Blood Volume Distribution 533
LUNDGREN Y B FOLKOW M HALLBACK and L WEISS Flow Resistance in Spontaneously Hypertensive Rats 93
MALMFORS T J de CHAMPLAIN L OLSON and CH SACHS Ontogenesis of Adrenergic Neurons 276
MÅRTENSSON A and CHR LINDQUIST Blink Reflex 149
MICHELI H and L A CARLSON PGE_1 on Fat Mobilizing Lipolysis 289
MICHELSEN K and P K M LUNDE Cortical Blood Flow in Rabbit Femur 39
NICKELS J Microelectrode Positioning 360
NICKELS J Miniature End Plate Potential 370
NILSSON L and B K SIESJO Anesthesia and Brain Metabolites 142
NILSSON L and B K SIESJO Anesthetics and Brain Metabolism 235
NJÅ A J K S JANSEN and L WALLOE Inhibitory Control of Stretch Receptors I 420
NJÅ A J K S JANSEN and L WALLOE Inhibitory Control of Stretch Receptors II 443
NOSAL R S A SLORACH and B UVNAS Degranulation and Histamine Release 215
ÖBERG B and S WHITE Stimulation of Vagus Afferents 383
ÖBERG B and S WHITE Circulatory Adjustment of Hemorrhage 395
OLSSON K E and B SALTIN Total Body Water and Muscle Glycogen 11
OLSON L J de CHAMPLAIN T MALMFORS and CH SACHS Ontogenesis of Adrenergic Neurons 276
OTTOSON D and G M SHEPHERD Synchronization of Spindle Activity 492
PAHLIN P E J KEWENTER and B STORM Nerve Stimulation on Jejunal Motility 353
PEDERSEN J I H T ANDERSEN E N CHRISTIANSEN and H J GRAV Brown Fat Thermogenesis 1
PETERSEN O H DNP and Secretory Potentials 117
PETERSON C and B DIAMANT ATP Induced Histamine Release 299
RASMUSSEN S N Renal Handling of Galactose 160
RASMUSSEN B and T B BARNETT Ventilatory Responses with Airway Resistance 538
RASMUSSEN F and N GYRD HANSEN PAH Clearance in Pigs 249
ROSELL S and B B FREDHOLM Noradrenaline and Adipose Tissue 404
ROSELL S and B B FREDHOLM PGE_1 in Adipose Tissue 450
SACHS CH and G JONSSON Noradrenaline Synthesis in Mouse Atrium 307
SACHS CH J de CHAMLAIN T MALMFORS and L OLSSON Ontogenesis of Adrenergic Neurons 276

SALTIN, B and K E OLSSON, Total Body Water and Muscle Glycogen 11
SCHMALBRUCH, H and F BUCHTHAL, Contraction Time of Twitches 378
SHEPHERD, G M and D OTTOSON, Synchronization of Spindle Activity 492
SIESJO, B K and L NILSSON, Anesthesia and Brain Metabolites 147
SIESJO, B K and L NILSSON, Anesthetics and Brain Metabolism 235
SILFVENIUS H, Projections from Interosseous Nerves 196
SJOSTRAND, N O and G SWEDIN, Potentiation of Seminal Vesicle Response 172
SLORACH S A, R NOSÁL and B UVNAS Degranulation and Histamine Release 215
SOMLO SZUCS, Z, P BJORNTORP, M KROTKIEWSKI and B LARSSON, Lipogenesis *in Vivo* 29
STAGE, L and B LJUNG, NA Control of Vascular Automaticity 131
STÅLHANDSKE, T, Liver Metabolism and Nicotine 222
STEPITA-KLAUCO, M and E GIACOBINI, Lithium and Stretch Receptor Neuron I 519
STEPITA-KLAUCO, M, E GIACOBINI and S HOVMARK, Lithium and Stretch Receptor Neuron II 528
STJARNE, L and Å WENNMALM, Preferential Noradrenaline Secretion 428
STORM, B, J KEWENTER and P-E PAHLIN Nerve Stimulation on Jejunal Motility 353
SUROWIAK, J and P E LINDAHL, Circadian Fluctuations of Acid Phosphatase 254
SVANBERG O, Incorporation of Cholesterol 45
SWEDIN, G and N O SJOSTRAND Potentiation of Seminal Vesicle Response 172
TREISTER, G and E H BÁRÁNY, Degeneration Mydriasis and Vasoconstriction 79
UVNAS, B, R NOSÁL and S A SLORACH, Degranulation and Histamine Release 215
WAALER B A, B FOLKOW and P GASKELL, Muscle Blood Flow during Heavy Exercise 61
VALLBO, Å B, Human Muscle Spindle Afferents 552
VALLBO, Å B and M KNIBESTOL, Mechanoreceptors in Human Glabrous Skin 178
WALLOE, L., J K S JANSEN and A NJÅ, Inhibitory Control of Stretch Receptors I 420
WALLOE, L, J K S JANSEN and A NJÅ Inhibitory Control of Stretch Receptors II 443
WEISS, L, B FOLKOW, M HALLBACK and Y LUNDGREN, Flow Resistance in Spontaneously Hypertensive Rats 93
WENNMALM, Å and L STJARNE, Preferential Noradrenaline Secretion 428
WHITE, S and B ÖBERG, Stimulation of Vagus Afferents 383
WHITE, S and B ÖBERG, Circulatory Adjustment of Hemorrhage 395
WIKSTROM, C, Phosphatase in Seminal Fluid 426

KUNGL BOKTR STHLM 1970

ACTA PHYSIOLOGICA SCANDINAVICA

ACTA PHYSIOL. SCAND

Vol. 80. INDEX

Fasc 1 (September 1970)

Thermogenesis of Brown Adipose Tissue Reflected in Mitochondrial Respiratory Control By H T Andersen E N Christiansen H J Grav and J I Pedersen 1

Variation in Total Body Water with Muscle Glycogen Changes in Man By K E Olsson and B Saltin 11

Blood Flow and Oxygen Extraction in the Cat Uvea at Normal and High Intraocular Pressures By A Alm and A Bill 19

Effects of Feeding States on Lipid Radioactivity in Liver Muscle and Adipose Tissue after Injection of Labelled Glucose in the Rat. By P Bjorntorp M Krotkiewski B Larsson and Z Somlo-Szucs 29

Determination of Cortical Blood Flow in Rabbit Femur by Radioactive Microspheres By P K M Lunde and K Michelsen 39

The Incorporation of Cholesterol into the Nervous System of the Newly Hatched Chick By O Svanberg 45

Plasma Skimming in the Intestinal Tract By M Jodal and O Lundgren 50

Blood Flow through Limb Muscles during Heavy Rhythmic Exercise By B Folkow P Gaskell and B A Waaler 61

Distribution of Choline Acetyltransferase in the Hearts of Mammals By J Ekstrom 73

Time Relations of Degeneration Mydriasis and Degeneration Vasoconstriction in the Rabbit Ear after Sympathetic Denervation Effect of Bretylium. By E H Bárány and G Treister 79

Background of Increased Flow Resistance and Vascular Reactivity in Spontaneously Hypertensive Rats By B Folkow M. Hallback Y Lundgren and L. Weiss 93

The Degeneration Contraction of a Sympathetically Innervated Smooth Muscle in the Rat after Reserpine Inhibition of Monoamine Oxidase or Tyrosine Hydroxylase By D Lundberg 107

The Effect of Dinitrophenol on Secretory Potentials Secretion and Potassium Accumulation in the Perfused Cat Submandibular Gland By O H Petersen 117

Exchange of Glucose between Plasma Brain Extracellular Fluid and Cerebral Ventricles in Cats and Effects of Intraventricular Acetazolamide and Insulin By H E Brond-dt 122

α-Adrenergic Excitatory Influences on Initiation and Conduction of Electrical Activity in the Rat Portal Vein By B Ljung and L. Stage 131

Short Communication

The Effect of Anesthetics on the Tissue Lactate Pyruvate Phosphocreatine ATP and AMP Concentrations and on Intracellular pH in the Rat Brain By L. Nilsson and B K. Siesjo 142

Fasc 2 (October 1970)

Effect of Hypotonic Mannitol and Saline Load on Diluting Capacity in Man By A Aperia, O Broberger and H Feychting 145

Mechanisms Involved in the Cat's Blink Reflex. By Chr Lindquist and A. Mårtensson 149

Renal Reabsorption and Metabolic Conversion of Galactose in the Cat By S N Rasmussen 160

Potentiation by Various Smooth Muscle Stimulants of an Isolated Sympathetic Nerve Seminal Vesicle Preparation from the Guinea Pig By N O Sjostrand and G Swedin 172

Single Unit Analysis of Mechanoreceptor Activity from the Human Glabrous Skin By M Knibestol and A B Vallbo 178

Projections to the Cerebral Cortex from Afferents of the Interosseous Nerves of the Cat By H Silfvenius 196

Quantitative Correlation between Degranulation and Histamine Release Following Exposure of Rat Mast Cells to Compound 48/80 *in vitro* By R Nosál, S A Slorach and B Uvnäs 215
Effects of Increased Liver Metabolism of Nicotine on Its Uptake, Elimination and Toxicity in Mice By T Stålhandske 222
The Effect of Anesthetics upon Labile Phosphates and upon Extra- and Intracellular Lactate, Pyruvate and Bicarbonate Concentrations in the Rat Brain By L. Nilsson and B K Siesjo 235
Acetylation of p-Aminohippuric Acid in the Kidney Renal Clearance of p-Aminohippuric Acid and N⁴-Acetylated p-Aminohippuric Acid in Pigs By N Gyrd-Hansen and F Rasmussen 249
The Circadian Fluctuations of the Amount of Free Phosphate and of the Activity of Acid Phosphatase in the Kidneys of Mice and the Effect of UV Radiation upon this Rhythm By P E Lindahl and J Surowiak 254
Antagonism by Calcium of the Inhibitory Action of Prostaglandin E_2 on Sympathetic Neurotransmission in the Cat Spleen By P Hedqvist 269
Ontogenesis of Peripheral Adrenergic Neurons in the Rat Pre- and Postnatal Observations By J de Champlain T Malmfors L. Olson and Ch Sachs 276

Fasc 3 (November 1970)

Effect of Prostaglandin E_1 on Fat Mobilizing Lipolysis in Rat Adipose Tissue in Relation to the Nutritional Condition By L. A Carlson and H Micheli 289
The Relationship between Arylesterase Activity and Growth in Rats By K B Augustinsson and B Henricson 295
The Effect of Glucose on Histamine Release from Isolated Rat Peritoneal Mast Cells Induced by Adenosine 5 Triphosphate By B Diamant and C. Peterson 299
Synthesis of Noradrenaline from 3 4 Dihydroxyphenylalanine (DOPA) and Dopamine in Adrenergic Nerves of Mouse Atrium — Effect of Reserpine Monoamine Oxidase and Tyrosine Hydroxylase Inhibition By G Jonsson and Ch Sachs 307
Effects of Some Adrenergic Neuron Blockers Related Quaternary Ammonium Compounds and Guanidine Derivatives on Degenerating Adrenergic Nerves in the Conscious Rat By D Lundberg 323
Cytoplasmic Distribution of Endogenous and Exogenous 5-Hydroxytryptamine in Rat Peritoneal Mast Cells By S E Jansson 345
The Effect of Periarterial Nerve Stimulation on the Jejunal and Ileal Motility in Cat By J Kewenter P E Pahlin and B Storm 353
Localization of a Microelectrode Tip in Muscle Cell A light and electron microscopic study By J Nickels 360
Correlation between the Rise Time of Miniature End Plate Potential and the Distance of the Recording Microelectrode from the Myoneural Junction By J Nickels 370
Contraction Times of Twitches Evoked by H Reflexes By F Buchthal and H. Schmalbruch 378
Circulatory Effects of Interruption and Stimulation of Cardiac Vagal Afferents By B Öberg and S White 383
Th[illegible]
39[illegible]
Fa[illegible]
and S Rosell 404
In Vitro Assay of the Stimulatory Effect of Hypoxic Serum on Cellular Proliferation in the Erythron By E Kivilaakso

Studies on the Action of Various Cyclic Nucleotides and Their Metabolites on the Synthesis of Corticosterone *in Vitro* By P BIECK H VAPAATALO and E WESTERMANN 28A
Serum Non Esterified Fatty Acids and Plasma Glycerol as Indicators of Fat Mobilization in Pregnant Sheep Subjected to Cold Stress By N ASTRUP, A ALLIE J F NEDKVITNE and W VELLE 30A
Dynamics of Fluid Transfer between the Intra and Extravascular Compartments during Exercise By J LUNDVALL S MELLANDER H WESTLING and T WHITE 31A
Increased Forearm Blood Flow during Ketoacidosis in Man By S L NIELSEN 32A
Physiological Interrelations during Intermittent and Continuous Exercise with the Same Average Power Output in Man By R H T EDWARDS C M HESSER I JACK A MELCHER A M McNINCH R D W WELLS and O WIGERTZ 33A
Hyperpolarizing Actions of Noradrenaline in Spinal Motoneurones By I ENGBERG and A THALLER 34A
Is Interruption of the Nigro Striatal Dopamine System Producing the Lateral Hypothalamus Syndrome ? By U UNGERSTEDT 35A
Autonomic Innervation of the Smooth Muscle Sphincter in the Swimbladder of a Fish (*Gadus morrhua*) By S NILSSON 36A
Differences in Effects of α Methyltyrosin on Various Peripheral Noradrenal ne Stores of the Rat By G SWEDIN 37A
Interference of Membrane Pump Blocking and α Receptor Blocking Drugs with Noradrenaline Release from Isolated Tissues By L O FARNEBO and B HAMBERGER 38A
Studies on Ribonucle c Acids of the Mammary Gland of Pregnant and Lactating Rabbits 39A
40A
41A
Changes in Sp nal Excitability during an Auditory Vertex Response in Man By H FRUHSTORFER T JARVILEHTO J LUMIO and P SOVERI 42A
Response Patterns of Human Muscle Sp ndle Endings to Isometric Voluntary Contractions By Å B VALLBO 43A
nlamo Cortical Rhythmicity during Reversible Visual Deafferentation By S ANDERSSON E HOLMGREN and T IVARSSON 44A
nfluence of Plasma Hyperton city on Blood Viscosity Studied *in Vitro* and in an Isolated Vascular Bed By S N RASMUSSEN 45A
Efflux of Prostaglandins in Lymph from Scalded Tissues By E ÄNGGÅRD G ARTURSON and C E JONSSON 46A
The Permeability of the Blood Brain Barrier to Lactate and Pyruvate By C CRONE and S C SORENSEN 47A
Local Regulation of Vascular Cross Section dur ng Changes in Femoral Arterial Blood Flow in Dogs By F KIIL, M LIE and O M SEJERSTED 48A

Supplements

Supplementum 347 Effect of Posture on Circulation and Respiration at Rest and during Exercise in Heart Disease By H ÅSTROM
Supplementum 348 Growth Characteristics of Adrenergic Nerves in the Adult Rat By L OLSON and T MALMFORS
Rat Portal Vein as Related to
MALMFORS and L OLSSON
Response to Nerve Stimulation in Rat Portal Vein *in vitro* By J HAGGENDAL B JOHANSSON J JONASON and B LJUNG

Nervous and Myogenic Mechanisms in the Control of a Vascular Neuro-effector System By B LJUNG
Supplementum 350 On the Relation between Blood Pressure and Blood Flow in the Canine Brain with Particular Regard to the Mechanism Responsible for Cerebral Blood Flow Autoregulation By B EKSTROM JODAL
Supplementum 351 Factors Influencing the Autonomic Component of the Defence Reaction By B LISANDER
Supplementum 352 Postnatal Development of Spinal Anterior Horn Neurones in Normal and Undernourished Rats A Quantitative Cytochemical Study By M HALTIA
Supplementum 353 Contractility of the Isolated Human Fetal Heart Influence of contraction rate acid base parameters and a local anaesthetics By G GENNSER K E ANDERSSON and F NILSSON
Supplementum 354 Studies on the Sympathetic Regulation of Circulation and Metabolism in Isolated Canine Subcutaneous Adipose Tissue By B B FREDHOLM

INDEX AUCTORUM

AARSETH, P, Pulmonary Blood Volume 459
ALM, A and A BILL, Oxygen Extraction in Uvea 19
ANDERSEN, H T, E N CHRISTIANSEN, H J GRAV and J I PEDERSEN, Brown Fat Thermogenesis 1
APERIA, A, O BROBERGER and H FEYCHTING, Hypotonic Mannitol and Saline Load 145
ÅSTRÖM, A, Potentiating Action of DMI 510
AUGUSTINSSON, K -B and B HENRICSON, Arylesterase Activity and Growth 295
BÁRÁNY, E H and G TREISTER, Degeneration Mydriasis and Vasoconstriction 79
BARNETT, T B and B RASMUSSEN Ventilatory Responses with Airway Resistance 538
BILL, A and A ALM, Oxygen Extraction in Uvea 19
BJÖRNTORP, P, M KROTKIEWSKI, B LARSSON and Z SOMLO SZUCS Lipogenesis *in Vivo* 29
BROBERGER, O, A APERIA and H FEYCHTING, Hypotonic Mannitol and Saline Load 145
BRØNDSTED, H E, Brain Glucose, Acetazolamide and Insulin 122
BUCHTHAL, F and H SCHMALBRUCH, Contraction Time of Twitches 378
CARLSON, L A and H MICHELI PGE_1 on Fat Mobilizing Lipolysis 289
de CHAMPLAIN J T MALMFORS, L OLSON and CH SACHS, Ontogenesis of Adrenergic Neurons 276
CHRISTIANSEN, E N, H T ANDERSEN, H J GRAV and J I PEDERSEN, Brown Fat Thermogenesis 1
DIAMANT, B and C PETERSON, ATP Induced Histamine Release 299
EKSTRÖM, J, Choline Acetyltransferase in the Heart 73
FEYCHTING, H, A APERIA and O BROBERGER Hypotonic Mannitol and Saline Load *145*
FOLKOW, B, P GASKELL and B A WAALER, Muscle Blood Flow during Heavy Exercise *61*
FOLKOW, B, M HALLBÄCK, Y LUNDGREN and L. WEISS Flow Resistance in Spontaneously Hypertensive Rats 93
[illegible] 567
[illegible] 401
[illegible] 450
GASKELL, P, B FOLKOW and B A WAALER, Muscle Blood Flow during Heavy Exercise 61
GIACOBINI, E and M STEPITA-KLAUCO, Lithium and Stretch Receptor Neuron I 519
GIACOBINI, E, S HOVMARK and M STEPITA KLAUCO Lithium and Stretch Receptor Neuron II 528
GRAV, H J, H T ANDERSEN E N CHRISTIANSEN and J I PEDERSEN Brown Fat Thermogenesis 1
GYRD HANSEN, N and F RASMUSSEN, PAH Clearance in Pigs 249
HALLBÄCK, M, B FOLKOW Y LUNDGREN and L WEISS, Flow Resistance in Spontaneously Hypertensive Rats 93
HEDQVIST, P Prostaglandin Antagonism by Calcium 269
HENRICSON, B and K B AUGUSTINSSON, Arylesterase Activity and Growth *295*
HERBAI, G, Sulfate Uptake into Cartilage 470
HERBAI, G and U LINDAHL *Biosynthesis* of Chondroitin Sulfate 502
HOLSTEIN, B, Histamine Metabolism in *Myxine* 433
HOVMARK, S, E GIACOBINI and M STEPITA KLAUCO Lithium and Stretch Receptor [illegible] 528
[illegible] ors I 420
[illegible] ors II 443
[illegible] 345
[illegible] 50
JODAL, M and O [illegible] 533
JODAL, M and O LUNDGREN Intestinal Blood Volume Distribution

JONSSON G and CH SACHS Noradrenaline Synthesis in Mouse Atrium 307
KARLSSON J and B B FREDHOLM Stimulated Fat Metabolism 567
KEWENTER J P F PAHLIN and B STORM Nerve Stimulation on Jejunal Motility 353
KIVILAAKSO E. Mitotic Stimulator in Hypoxic Serum 412
KIVILAAKSO E RNA Synthesis and Mitotic Regulation 436
KNIBESTOL M and Å B VALLBO Mechanoreceptors in Human Glabrous Skin 178
KROTKIEWSKI M P BJORNTORP B LARSSON and Z SOMLO SZÜCS Lipogenesis *in Vivo* 29
LARSSON B P BJORNTORP, M KROTKIEWSKI and Z SOMLO SZÜCS Lipogenesis *in Vivo* 29
LINDAHL P E and J SUROWIAK, Circadian Fluctuations of Acid Phosphatase 254
502
149
131
107
LUNDBERG D Neuron Blockers and Degeneration Contraction 323
LUNDBERG D Colchicine Induced Delay of the Degeneration 430
LUNDE P K M and K MICHELSEN Cortical Blood Flow in Rabbit Femur 39
LUNDGREN O and M JODAL, Intestinal Plasma Skimming 50
LUNDGREN O and M JODAL, Intestinal Blood Volume Distribution 533
LUNDGREN Y B FOLKOW M HALLBACK and L WEISS Flow Resistance in Spontaneously Hypertensive Rats 93
MALMFORS T J de CHAMPLAIN L. OLSON and CH SACHS Ontogenesis of Adrenergic *Neurons* 276
149
289
33
NICKELS J Microelectrode Positioning 360
370
142
235
NJÅ A J K. S JANSEN and L WALLOE Inhibitory Control of Stretch Receptors I 420
NJÅ A J K S JANSEN and L WALLOE Inhibitory Control of Stretch Receptors II 443
NOSÁL R S A SLORACH and B UVNAS Degranulation and Histamine Release 215
ÖBERG B and S WHITE Stimulation of Vagus Afferents 383
ÖBERG B and S WHITE Circulatory Adjustment of Hemorrhage 395
OLSSON K E and B SALTIN Total Body Water and Muscle Glycogen 11
OLSON L. J de CHAMPLAIN T MALMFORS and CH SACHS Ontogenesis of Adrenergic Neurons 276
OTTOSON D and G M SHEPHERD Synchronization of Spindle Activity 492
PAHLIN P E J KEWENTER and B STORM Nerve Stimulation on Jejunal Motility 353
PEDERSEN J I H T ANDERSEN E N CHRISTIANSEN and H J GRAV Brown Fat Thermogenesis 1
17
9
0

ROSELL S and B B FREDHOLM PGE_1 in Adipose Tissue
SACHS CH and G JONSSON Noradrenaline Synthesis in Mouse Atrium
SACHS CH J de CHAMLAIN T MALMFORS and L OLSSON Ontogenesis of Adrener Neurons

SALTIN, B and K E OLSSON Total Body Water and Muscle Glycogen 11
SCHMALBRUCH, H and F BUCHTHAL Contraction Time of Twitches 378
SHEPHERD, G M and D OTTOSON, Synchronization of Spindle Activity 492
SIESJO B K and L NILSSON, Anesthesia and Brain Metabolites 142
[illegible] 235
[illegible] 196
[illegible] 172
SLORACH, S A, R NOSÁL and B UVNÄS Degranulation and Histamine Release 215
SOWLO SZUCS, Z, P BJORNTORP, M KROTKIEWSKI and B LARSSON, Lipogenesis in Vivo 29
STAGE, L and B LJUNG, NA Control of Vascular Automaticity 131
STÅLHANDSKE, T, Liver Metabolism and Nicotine 222
STEPITA KLAUCO, M and E GIACOBINI Lithium and Stretch Receptor Neuron I 519
STEPITA KLAUCO, M, E GIACOBINI and S HOVMARK Lithium and Stretch Receptor Neuron II 528
STJARNE L and Å WENNMALM Preferential Noradrenaline Secretion 428
STORM B, J KEWENTER and P E PAHLIN, Nerve Stimulation on Jejunal Motility 353
SUROWIAK, J and P E LINDAHL, Circadian Fluctuations of Acid Phosphatase 254
SVANBERG, O, Incorporation of Cholesterol 45
SWEDIN, G and N O SJOSTRAND, Potentiation of Seminal Vesicle Response 172
TREISTER G and E H BÁRÁNY, Degeneration Mydriasis and Vasoconstriction 79
UVNAS B R NOSÁL and S A SLORACH, Degranulation and Histamine Release 215
WAALER, B A, B FOLKOW and P GASKELL, Muscle Blood Flow during Heavy Exercise 61
VALLBO, Å B, Human Muscle Spindle Afferents 552
VALLBO, Å B and M KNIBESTOL, Mechanoreceptors in Human Glabrous Skin 178
WALLOE, L, J K S [illegible] 420
WALLOE, L, J K S [illegible] 443
WEISS, L, B FOLKOW, [illegible]
ly Hypertensive Rats 93
WENNMALM Å and L STJARNE, Preferential Noradrenaline Secretion 428
WHITE, S and B ÖBERG, Stimulation of Vagus Afferents 383
WHITE, S and B ÖBERG, Circulatory Adjustment of Hemorrhage 395
WIKSTROM, C, Phosphatase in Seminal Fluid 426

KUNGL BOKTR. STHLM 1970

Brown adipose tissue (BAT) is a major source of heat liberated by non-shivering thermogenesis. Consequently, much research has been carried out with this tissue and several hypotheses have been advanced in support of one or the other of the mechanisms referred to above. The first alternative has been advocated by Ball and Jungas (1961) who proposed an increased utilization of ATP by recycling of fatty acids. Recent experiments have shown this explanation to be *untenable* (Kornacker and Ball 1968).

Girardier, Seydoux and Clausen (1968) have suggested ADP generation through the operation of a sodium-potassium dependent ATPase in the cell membrane. The activation of such a mechanism was demonstrated as variations in the membrane potential of BAT cells. However, the quantitative importance of this phenomenon has not been established. It may be concluded, with respect to the first alternative, that no cytosol reaction known consumes ATP with sufficient rapidity to maintain respiration at a high rate over an extended period of time.

The second alternative has been investigated more closely, yet the mechanism underlying BAT *thermogenesis remains obscure*.

Principally, the physiological significance of an "uncoupling hypothesis" cannot be established unless a decrease in phosphorylation efficiency is demonstrated as a response to physiological cold stimuli. Also, it is necessary to show that such a bio-adaptive process is reversible. However, it is not always acceptable to present *in vitro* observations of low P/O ratios as evidence of uncoupling of phosphorylation from respiration under *in vivo* conditions. The respiratory control ratio, i.e. the rate of oxygen uptake in the presence of ADP divided by the rate of oxygen uptake in the absence of ADP, has to be studied simultaneously.

Oxidative phosphorylation and mitochondrial respiratory control are parallel phenomena related to the conservation of energy in bio-systems. Both reflect the degree of coupling of phosphorylation to respiration, yet they are independent to such an extent that they may be separated experimentally. Thus, Hoch and Lipmann (1954) were able to obtain high P/O ratios in the absence of respiratory control. When low respiratory control ratios are observed it is essential that the P/O ratios are determined as true incorporation of P into ATP, for instance by the glucose-hexokinase reaction, since the polarographic method may be used with tightly coupled mitochondria only. *Thus, loose coupled systems give erroneously low values for the* ratio P/O.

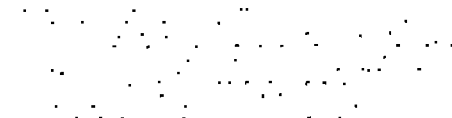
It has been shown previously that BAT mitochondria from newborn guinea pigs exhibited lower P/O ratios than weaned animals when the *in vitro* conditions induced lack of respiratory *control* in both age groups (Christiansen, Pedersen and Grav 1969). More recently it has been reported that when the *in vitro* conditions favoured maximal P/O ratios in all age groups the respiratory control ratio was markedly lowered in the neonatal stage whereas no such effect was detected in the older animals (Grav, Pedersen and Christiansen 1970).

These results are consistent with the hypothesis that BAT thermogenesis in newborns is associated with a decrease in mitochondrial ATP production. *The purpose of*

the investigation reported here was to learn whether cold exposure would cause a similar response in older animals. A preliminary account of this work has appeared in this journal (Andersen *et al.* 1969).

Material and methods

Young guinea pigs of the Pir/Srr/c strain (State Institute of Public Health, Oslo, Norway) were used in the experiments. They were maintained at 21—26° C during the first 3—4 weeks of life. At the age of 21 or 30 days the animals were divided into two groups. One of the groups remained at an ambient temperature of 21—26° C. The animals of the other group was exposed to an environmental temperature of 6° C for variable periods of time. Some of the



respiratory control were performed as previously described by Grav *et al.* (1970).

Skeletal muscle mitochondria were prepared according to Ernster and Nordenbrand (1967) and suspended in 0.33 *M* sucrose—1 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid pH 7.2.

The composition of the reaction media used for determinations of P/O ratios is given in the legends to Fig. 2 and Table II. Respiratory control values were measured using the Yellow Springs Instruments Oxygen Monitor with the medium described in the legend to Fig. 4.

Results

Effects of temperature on brown adipose tissue

The wet weight of BAT decreased during cold stress (Table I). No reversal of this BAT loss was observed when the period of cold exposure was prolonged to 60 days at which stage an amount of 0.49 ± 0.13 g BAT/100 g b.w. was measured.

Upon cold exposure the colour of the BAT changed from yellowish to a brown colour similar to that seen in the newborn guinea pig.

Very high yields of BAT mitochondria were obtained from cold exposed animals, the average figure being 4.5 times that found in guinea pigs kept at the temperature range of 21—26° C for a period of 6—20 days.

Energy conserving mechanism of brown adipose tissue mitochondria altered by thermal stimuli

The ability of BAT mitochondria to carry out oxidative phosphorylation and their level of respiratory control vary markedly with the *in vitro* conditions under which they are studied (Grav *et al.* 1970). In Fig. 1 is illustrated the way in which the P/O ratio and the degree of respiratory control exhibited by BAT mitochondria from newborn guinea pigs vary with pH. Obviously, determination of ATP production associated with oxygen consumption offer the least sensitive measurement of

TABLE I Weight variations in interscapular brown adipose tissue (BAT) and corresponding yields of mitochondrial protein during cold stress
Numbers in parentheses relates the number of different animals used in the various experiments [1]

Temperature °C	Time of exposure days	g BAT/100 g body weight	Mitochondrial protein mg/g BAT
21—26	6—20	0 99±0 27 (15)	0 64±0 51 (15)
6	6—20	0 68±0 15 (11)	2 96±0 96 (11)
21—26	60	0 90 (2)	0 01 (1)
6	60	0 49±0 13 (4)	3 05±0 77 (4)

[1] *Animals were 20 or 30 days old before cold exposure was initiated*

chondrial energy conserving activity since the P/O ratio hardly differ over the pH interval of 6 0—7 5 The respiratory control ratio on the other hand shows a very well defined pH optimum at pH 7 0 In the experiments illustrated in Fig 2 and 3 the *in vitro* conditions chosen were such that the phosphorylation capacity was retained but without any detectable respiratory control

Mitochondria from cold stressed animals showed a markedly reduced phosphorylation efficiency (Fig 2) When cold exposed animals were returned to a thermoneutral environment their mitochondrial oxidative phosphorylation returned to the P/O range characteristic of the warmer surroundings (Fig 3) In these experiments there were no differences in respiratory control ratios between mitochondria from the various animal groups

Under similar conditions the *in vitro* oxidative phosphorylation of mitochondria prepared from liver and muscle remained unaffected in temperature stressed animals In fact after 2 months in the cold muscle mitochondria yielded a P/O ratio of 2 72 with pyruvate-malate as substrate versus 2 22 for the warm control The corresponding values for the liver were 2 71 and 2 27 All four mitochondrial preparations showed evidence of respiratory control

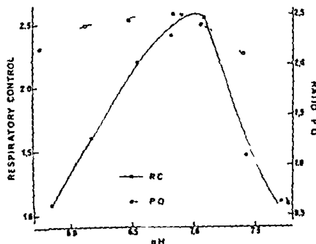


Fig 1 Influence of pH on oxidative phosphorylation and respiratory control ratios of BAT mitochondria from newborn guinea pigs respiring on endogenous substrate Experimental conditions as described in legend to Table II (P/O determinations) and Fig 4 trace F (respiratory control ratio)

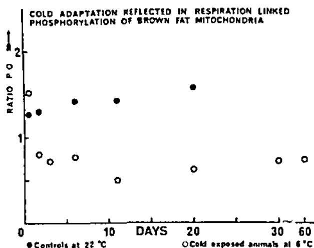


Fig 2 Effect of cold stress on oxidative phosphorylation in BAT mitochondria

Components of the incubation medium were 10 mM KH_2PO_4 buffer pH 7·4 with 100 mμCi ^{32}P per μmole P_i 1 per cent bovine serum albumin (Mann Research Laboratories fatty acid poor), 240 mM sucrose 30 μM cytochrome c 2 mM ATP 7·5 mM $MgCl_2$ 60 mM HEPES (Calbiochem) buffer pH 7·4 50 mM glucose, 11 units/ml hexokinase (Sigma Chemical Company) in a total volume of 2·5 ml Mitochondrial protein was 0·3—0·5 mg/ml and 5 mM pyruvate—5 mM malate (containing 0·25 mM NAD) was used as substrate The polarographic experiments were started by the addition of substrate followed by P_i + hexokinase Incorporated phosphate was determined by the isotope distribution method and related to the equivalent oxygen consumption as measured from polarographic traces The animals were aged 30 days prior to exposure Each point represent the mean result with 1—3 animals mitochondria being prepared from each animal

In another series of experiments the *in vitro* conditions were chosen so as to favour observations of the degree of respiratory control as well as that of oxidative phosphorylation The results are presented in Table II Under these conditions no consistent differences were detected in the P/O ratios between mitochondria from cold exposed animals and those from their controls This observation was made with pyruvate malate and confirmed by using succinate as substrate in conjunction with the inhibitor rotenone thus eliminating endogenous respiration Determinations of the P/O ratio with endogenous substrates only gave similar results With exogenous substrates the respiratory rates measured in mitochondria from cold exposed animals differed markedly from those of their controls the respiratory rates of the former

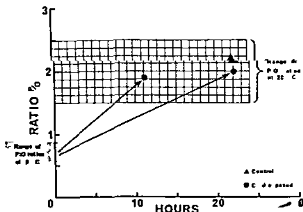


Fig 3 Mitochondrial recoupling in cold exposed animals returned to a warm environment The animals were exposed to 6° C for 12—17 days before being transferred to a warm environment The experimental conditions were as described in the legend to Fig 2

TABLE II Oxidative phosphorylation ratios (P/O) and respiratory rates of brown adipose tissue mitochondria from animals exposed to different temperatures [1]

Temperature	Substrates				Endogenous respiration	
	Pyruvate malate		Succinate			
	P/O	ngatoms O/ min/mg protein	P/O	ngatoms O/ min/mg protein	P/O	ngatoms O/ min/mg protein
21—26	2 51	40 3	1 39	38 0	2 95	41 7
	2 32	36 3	1 40	66 9	2 44	47 4
6° (10 days)	2 77	95 0	1 47	143 5	2 72	47 4
	2 72	95 5	1 26	114 2	2 58	37 6
			1 41	114 5		
6° (10 days followed by 21—26						
12 hours			1 35	115 6		
24 hours	3 02	72 1				
36 hours			1 22	99 3		

[1] The basic medium was that described in the legend to Fig 4 and containing 2 mM ATP The reactions were started by adding substrate (5 mM pyruvate +5 mM malate or 9 6 mM succinate +0 6 μM rotenone) followed by the addition of 5 mM $KH_2PO_4+{}^{32}P$+hexokinase after which *the respiratory rate was determined The P/O ratio was determined by relating the incorporated* phosphate to the equivalent oxygen consumption

being approximately twice those of the latter Mitochondria taken from animals returned to the warm environment for 12 36 hrs after cold exposure showed intermediate values

Consistent and characteristic differences in mitochondrial respiratory control appear in the polarographic traces presented in Fig 4 The respiratory control pattern of BAT mitochondria from animals kept in the warm is similar to that of mitochondria from other organs and tissues when exogenous or endogenous substrates serve as energy sources In these experiments (Fig 4 traces A C and E) respiration could be repeatedly stimulated if small amounts of ADP were added in the presence of inorganic phosphate The respiratory rate would return to the original level when the supply of ADP was exhausted ADP stimulated respiration was strongly impeded by oligomycin (Oligo This inhibition was relieved again when the uncoupling agent carbonyl cyanide phenylhydrazone (CCP) was added

Respiration of mitochondria from cold stressed animals is stimulated with exogenous substrates (Fig 4 traces B and D) and further enhanced with P but affected only to a slight degree by ADP with pyruvate-malate or not at all using succinate with rotenone After addition of ADP respiration did not level off with time indicating absence of phosphate acceptor control The respiratory rates with exogenous substrates were threefold higher than those of mitochondria from control animals Even in the absence of ADP the respiratory rates were 2 3 times higher with cold stressed animals than with the controls respiring in presence of ADP The oligomycin sensitivity in mitochondria from cold exposed animals was significantly reduced as

PYRUVATE–MALATE

SUCCINATE

ENDOGENOUS

Fig 4 Respiratory patterns of brown adipose tissue mitochondria from cold exposed and control animals The incubation mixture contained the following components in a total volume of 2 5 ml 105 mM sucrose 50 mM glucose 40 mM HEPES buffer pH 6 8 2 % bovine serum albumin 1 mM $MgCl_2$ 2 mM EDTA mitochondria (1 00 2 16 mg protein) and where succinate served as substrate 0 6 μM rotenone

4 mM pyruvate—4 mM malate or 9 6 mM succinate 5 mM KH_2PO_4 (P_i), 0 1 mM ADP 1 μg oligomycin and 50 μM CCP were added as indicated at temperature 25° C The numbers represent the specific respiratory rates at the various slopes of the curve (ngatoms oxygen per min per mg mitochondrial protein) Traces A C and E are the controls B D and F the cold exposed animals Trace G represents an experiment parallel to trace F where the mito[illegible] were preincubated with 1 mM ATP

TABLE III Respiratory control ratios with brown adipose tissue mitochondria from animals exposed to different temperatures. Each figure represents mean of the number of experiments given in parentheses The experimental conditions were those described in the legend to Fig 4

Temperature exposure	Substrates		Endogenous respiration
	Pyruvate — malate	Succinate	
21—26°	2 58 (6)	1 93 (4)	3 55 (2)
6° 1 day		*1 97 (1)*	
2 days		1 59 (3)	
10—12 days	1 47 (4)	*1 02 (5)*	*1 61 (2)*
6° (10 days) followed by 21—26°			
12 hours		1 30 (1)	
36 hours		1 84 (1)	

compared with that of the controls more so using succinate with rotenone as substrate than with pyruvate malate CCP stimulation of the oligomycin inhibited respiration was similarly reduced when succinate with rotenone was added but was unaffected in experiments carried out with pyruvate malate as substrate

Tracings obtained with mitochondria from cold stressed animals respiring on endogenous substrates showed an initial stimulation with a small amount of ADP without subsequent return to the original rate (Fig 4 trace F) indicating that this amount of ADP was sufficient to launch the system into a continuously active state We interpret this as activation of endogenous fatty acids the ATP required for this activation being generated from exogenous ADP via substrate level phosphorylation It should be noted that even in this case there is a small inhibition of respiration by oligomycin and subsequent stimulation by CCP suggesting a certain degree of respiratory control Moreover the extent of oligomycin inhibition has been shown to be a function of the concentration of ADP present increasing with rising ADP concentration (Pedersen unpubl obs) This may apply also to the oligomycin inhibition seen with *pyruvate malate (Fig 4 trace B)*

The maximal respiratory rates of mitochondria respiring on endogenous substrates were higher for the controls than for cold exposed animals This finding may be due to lack of sufficient ATP to support fatty acid activation in the cold stressed animals That ATP so affects the system that the maximal respiratory rates reach the level typical of mitochondria from control animals supports this conclusion ATP moreover transforms the respiratory pattern into one exhibiting respiratory control (Fig 4 trace G

Table III summarises the results of respiratory control measurements of a larger number *of animals based on experiments such as those shown in Fig 4* The decrease in respiratory control ratios which occurs in cold exposure is reproducible with all substrates. Mitochondria of animals returned from a cold to a warm environment re-establish respiratory control as is indicated in respiration experiments using succinate (with rotenone as substrate

Discussion

Recent studies of BAT mitochondria from newborn guinea pigs have revealed a decreased incorporation of P_i into ATP in the neonatal stage as compared to the values found in older animals (Pedersen, Christiansen and Grav 1968, Rafael, Klaas and Hohorst 1968 Christiansen *et al* 1969) Moreover, it has been reported that this loose coupling of phosphorylation to respiration in the newborn may be detected on two different levels, by determinations of the P/O ratio *and* by measurements of respiratory control ratios (Grav *et al* 1970) All of these observations have been interpreted in terms of a physiological response to cold stimulation

The results presented in this paper provide additional support in favour of the hypothesis that BAT thermogenesis depends upon a loose coupling of phosphorylation to respiration since BAT mitochondria from weaned guinea pigs have been shown to develop phosphorylation deficiency during cold exposure Also, the process is a reversible one as has been demonstrated in cold exposed animals subsequently returned to a thermoneutral environment Under the experimental conditions described in Fig 2 exposure of the animals to a cold environment (6° C) is accompanied by a significant lowering of BAT mitochondrial phosphorylation capacity Reversibility of this process is illustrated in Fig 3 When *in vitro* conditions elicit the maximal potential of phosphorylation capacity of the mitochondria, variations in P/O ratios become suppressed (Table II) In such circumstances cold exposure is associated with lowering of the respiratory control ratios (Fig 4, Table III) This suggests a loosening of the coupling With exogenous substrates, loose coupling was accompanied by a 3-fold increase in the respiratory rates (Table II, Fig 4), an observation which agrees well with the increments in the corresponding tissue Q_{O_2} values as measured by others (Smith and Horwitz 1969) With mitochondria of cold stressed animals respiring without added substrates, on the other hand rates of oxygen uptake appeared to be limited by the endogenous level of available ATP and by substrate (Fig 4 curve F)

That ATP influences both respiratory control and the respiratory rate is apparent from Fig 4, curve G It is reasonable to assume, therefore, that cold stress is associated with a lowering of the endogenous level of mitochondrial ATP Similar trends have been observed during the postnatal stage (Pedersen unpubl obs) According to this view the ATP endogenous to BAT mitochondria should be maintained during thermogenic phases of the tissue at a level below that which induces tightening of coupling but high enough to support activation of fatty acids Thus, an uncoupling effect by fatty acids may be a factor in the mechanism controlling such an equilibrium (Hittelman Lindberg and Cannon 1969)

Liu Frehn and LaPorta (1969) did not detect any variations in the phosphate acceptor control of BAT mitochondria from hibernating and non hibernating animals during cold exposure Their negative results are most likely due to the *in vitro* conditions used which have been shown to be noxious to the respiratory control mechanism (Grav *et al* 1970) Our own findings reported in this paper strongly indicate that t

degree of coupling of phosphorylation to respiration is not simply an effect that may be elicited by a chosen set of suitable *in vitro* conditions, but a physiologically regulated mechanism reversibly responsive to thermal stimuli Furthermore the reversible loose coupling of BAT mitochondria may not be governed by on off control only It appears from the respiratory control values (Table III) that the degree of coupling of phosphorylation to respiration in the recovery period after cold exposure increases gradually Thus, it seems likely that the uncoupling and recoupling processes may be regulated according to a principle that do not only take the temperature stress into account but also the time of exposure

The authors would like to thank Wenche Delingsby and Gerd Thune Jorgensen for their excellent technical assistance

This work has been supported by A/S Freia Chocolade Fabriks Arbeidsfond for Ernærings forsking

References

ANDERSEN H T E N CHRISTIANSEN H J GRAV and J I PEDERSEN Brown fat thermogenesis reflected in mitochondrial respiration *Acta physiol scand* 1969 *76* 12A

BALL E G and R L JUNGAS On the action of hormones which accelerate the rate of oxygen consumption and fatty acid release in rat adipose tissue in vitro *Proc natn Acad Sci USA* 1961 *47* 932—941

BUTOW R A and E RACKER On the mechanism of respiratory control *J gen Physiol* 1965 *49* 149 162

CHRISTIANSEN E N J I PEDERSEN and H J GRAV Uncoupling and recoupling of oxidative phosphorylation in brown adipose tissue mitochondria *Nature* (Lond) 1969 *222* 857—860

ERNSTER L and K NORDENBRAND *Skeletal muscle mitochondria* In "Methods in Enzymology Editors S P Colowick and N O Kaplan Acad Press New York 1967 *10* 86 94

GIRARDIER L J SEYDOUX and T CLAUSEN Membrane potential of brown adipose tissue *J gen Physiol* 1968 *52* 925 940

GRAV H J J I PEDERSEN and E N CHRISTIANSEN Conditions in vitro which affect respiratory control and capacity for respiration linked phosphorylation in brown adipose tissue mitochondria *European J Biochem* 1970 *12* 11 23

HITTELMAN K J O LINDBERG and B CANNON Oxidative phosphorylation and compartmentation of fatty acid metabolism in brown fat mitochondria *European J Biochem* 1969 *11* 183 19

HOCH F L and F LIPMANN The uncoupling of respiration and phosphorylation by thyroid hormones *Proc nat Acad Sci* (Wash) 1954 *40* 909—921

KERNACKER M S and E G BALL Respiratory processes in brown adipose tissue *J biol Chem* 1968 *243* 1638 1644

LIU C-C J I FRENCH and A D LAPORTA Liver and brown fat mitochondrial response to cold in hibernators and non hibernators *J appl Physiol* 1969 *27* 83—89

PEDERSEN J I E N CHRISTIANSEN and H J GRAV Respiration linked phosphorylation in mitochondria of guinea pig brown fat *Biochem biophys Res Commun* 1968 *32* 492—500

RAFAEL J D KLAAS und H J HOHORST Mitochondrien aus braunem Fettgewebe Enzyme und atmungsketten phosphorylierung während der prä und postnatalen entwicklung des interscapularen fettkörpers des meerschweinchens *Hoppe-Seyler's Z physiol Chem* 1968 *349* 1711 1724

SMITH R E and B A HOROWITZ Brown fat and thermogenesis *Physiol Rev* 1969 *49* 330 425

Acta physiol. scand. 1970. 80. 11—18

From the Department of Physiology, Gymnastik- och Idrottshögskolan and the Orthopedic Clinic, Karolinska institutet, Stockholm, Sweden

Variation in Total Body Water with Muscle Glycogen Changes in Man

By

Karl-Erik Olsson and Bengt Saltin

Received 10 January 1970

Abstract

Olsson, K. E. and B. Saltin. *Variation in total body water with muscle glycogen changes in man.* Acta physiol. scand. 1970. *80.* 11—18.

19 subjects performed prolonged heavy arm and leg exercise after which they had a protein and fat diet for three days. Thereafter they switched to a carbohydrate enriched diet during a 4 day period. The measurements were performed on the 3rd day and then repeated on the 7th day. The glycogen concentration in the thigh and the arm muscles was 4.5 and 2.6 g/kg wet muscle on the 3rd day and increased with the carbohydrate enriched diet to 19.9 and 16.9 g/kg [illegible]

Glycogen is bound with water in the liver, and figures of 2—4 g water per g glycogen have been given (Puckett and Wiley 1932, Fenn 1939). Pavy (1860) assumed that when glycogen was stored in muscle tissue some water was also deposited. Man's total muscle glycogen stores can be varied by physical exercise and carbohydrate enriched diet from 0—800 g (Bergström *et al.* 1967). If water is bound with glycogen in human muscle, body weight should change more with increasing glycogen stores than the above given 800 g. Depending upon how this water is bound to the glycogen another question of great interest is whether methods for determination of total body water measure this extra storage of water. The aim of the present study was therefore to measure body weight and total body water when the body's glycogen stores were first depleted and then refilled.

Material and methods

19 young healthy male physical education students were studied. Their age averaged 24 years, height 1[illegible]
Kidney [illegible]
weights [illegible]
tritium [illegible]

TABLE I Composition of the material with regard to age, maximal oxygen uptake, height Body weight, total body water and glycogen contents of muscle tissue are given before (Low) and after (High) filling muscle glycogen depots

No	Age yrs	Maximal oxygen uptake l/min	Height cm	Body weight Low kg	Body weight High kg	Total body water Low l	Total body water High l	Glycogen, g/kg wet muscle Low leg	Low arm	High leg	High arm
1	23	3.9	183	71.2	73.6	45.5	48.8	5.4		23.0	
2	24	4.0	181	89.8	92.2	53.0	54.8	7.7		22.0	
3	21	3.8	181	70.5	71.9	46.8	46.3	5.8	3.6	23.9	13.9
4	22	3.4	172	65.9	67.3	42.0	41.2	9.5	4.9	23.1	19.3
5	22	3.8	181	72.6	73.0	45.0	46.4	3.6	2.0	18.2	3.6
6	25	4.1	182	75.8	77.9	49.5	50.6	6.3		20.0	
7	25	4.4	190	79.0	81.5	46.5	49.1	1.6	1.3	24.5	21.2
8	25	4.3	180	72.3	75.8	44.7	47.5	5.0	2.5	16.9	13.9
9	26	3.4	173	71.4	73.3	43.8	44.9	5.7	2.0	14.2	12.9
10	24	4.4	185	75.4	78.2	47.3	51.5	3.4	1.3	28.4	24.4
11	24	3.5	179	72.0	74.8	44.3	47.3	1.1	2.9	26.0	25.9
12	22	3.4	180	63.8	67.5	38.2	43.1	5.2		24.9	
13	23	3.8	179	71.4	72.5	44.5	44.2	2.6		14.0	
14	24	4.6	177	73.4	75.9	48.1	50.5	5.2		14.8	
15	27	4.4	176	76.8	79.7	45.0	49.7	5.1		15.9	
16	28	4.2	183	79.9	82.4	45.2	48.5	3.8		12.5	
17	23	4.6	185	73.6	76.6	48.8	52.4	3.4		18.5	
18	24	4.9	190	77.4	80.9	50.4	52.8	2.9		19.3	
19	25	3.7	173	68.9	70.6	41.6	42.8	2.1		17.5	
mean	24	4.1	181	73.7	76.1	45.8	48.0	4.5	2.6	19.9	16.9
p				<0.001		<0.05		<0.001 leg <0.001 arm			

venously and blood samples were taken 3, 3.5 and 4 hrs later when the tracer substance was equilibrated in the total body water as was shown by Olsson and Plantin (1970) The tritium activity was counted in a liquid scintillation counting system after vacuum destillation of the blood samples Total body water was determined with a reproducability of ±4.0 per cent (S D) (Olsson 1970) and details about the method can be found in the same reference The tritium activity in the blood was measured daily in order to calculate the turn over rate of body water The specific activity of tritium in the body water decreased exponentially but was linear in a semi logarithmic plot after the short mixing phase (Haley *et al* 1951, Roberts *et al* 1958) Turn over rate is then defined as the λ value for the linear regression line Glycogen content in the muscle was determined on biopsy needle material with a method described by Hultman (1967) The biopsy was taken from the thigh (lateral portion of *m quadriceps femoris*) and also in 8 subjects from the shoulder muscle (*m deltoideus*) The error of the glycogen method determined on 2 different pieces from the same biopsy has been found to be ±6 per cent (S D) Determinations of the plasma volume were performed by injection of iodine tagged albumine (I^{131}) and samples were taken 10 and 20 min after the initial injection Sodium and potassium concentration in serum and urine were determined with an autoflame photometer technique and chloride concentration in serum and urine with Lehmann's method (1939) Creatinine in serum and urine was determined according to Folin's and Wu's modified method Technicon N method file N 11b

Procedure

In order to deplete the glycogen stores the subjects performed hard physical work for 2—4 hrs on the first day of the study which in most of the subjects included arm and leg exercise During the next 5 days the subjects had a special diet (3200 kcal) consisting of only fat and protein during the first 2 days and then of carbohydrates (2300 kcal) and protein for the remainder of the time Water was given in excess to the sensation of thirst every day from the

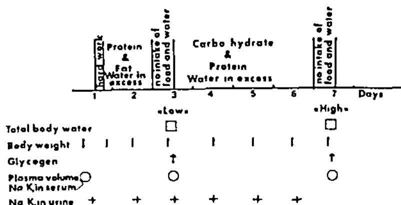


Fig 1 The experimental design

first to the seventh day of study Only limited physical effort was permitted during these 5 days Total body water, body weight and glycogen determinations were made twice, first when the glycogen values were expected to be lowest (day 3 = 'low") and secondly after the period with carbohydrate enriched diet when the values were expected to be highest (day 7 = high) (see Fig 1) Body weight and total body water were determined in the morning after the subjects had emptied their bowel and bladder and before any food or fluid intake During the determination of the "tritium space" (day 3 and 7) the subjects had no meals Body weight urine volume, serum and urine electrolytes were measured daily in ten of the nineteen subjects On these subjects blood samples were also taken daily and the specific activity of tritium of plasma water was determined Total water exchange could then be calculated from the turn over rate of tritium

Ordinary statistical methods were used and the P values refer to comparisons between intra individual differences (Snedecor 1956) All means are given ± the standard deviation (S D)

Results

The mean glycogen concentration in the thigh muscle in situation 'low" was 4 5 g/kg wet muscle (range 1 1—9 5) and in situation 'high" 19 9 g/kg (range 12 5—28 4) For the arm muscles the corresponding values were 2 6 g/kg wet muscle (range 1 3—4 9) and 16 9 (range 3 6—25 9) g/kg wet muscle (Table I) The increase in body weight between the two different occasions of investigation averaged 2 4 (range 1 1—3 8) kg The total body water volume varied from a mean of 45 8 l (range 38 2—53 0) in situation "low' to 48 0 l (range 41 2—54 8) in situation high" ($p < 0.05$)

Daily measurements of body weight in 10 of the subjects showed that the increase in body weight was very small during the period of combined fat and protein diet, mean 0 5 kg The greatest increase occurred when the subjects had the carbohydrate diet and 78 per cent of the total increase in body weight occurred during the last 2 days (days 5—7) of the experiment In the same group the urine volumes were largest during days 2—4, mean 1 6 l/day, and they then decreased during day 5 and 6 mean 1 1 l/day

The total volume of fluid intake, calculated as total water exchange from turn over rate of tritium was measured during days 3—6 and found to average 3 9 ±

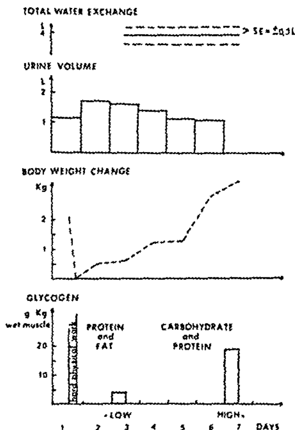


Fig 2 Mean values for 10 subjects during the course of the study lasting for 7 days In the top panel is the total water exchange ± S E The next panel gives the daily urine volumes The third panel gives the body weight changes The lowest panel indicates the variation in glycogen in the thigh

0 3 l/day (mean ± S E M) (Fig 2) The volume of fluid intake registered by the subjects during days 3 4 5 and 6 averaged 2 1, 2 5, 2 1, and 1 9 l/day, respectively

Fig 3 shows for the specially studied 10 subjects a comparison between plasma volume sodium potassium and chloride in serum in situations "low and high" The intra-individual differences between the values for these variables were not significant ($p > 0.05$) The ratio of K and Na in urine varied between 0 11 and 0 71 during the experimental period

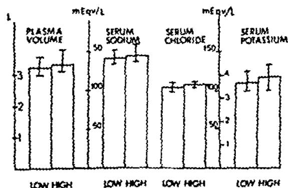


Fig 3 Mean values from 10 subjects of plasma volumes and serum electrolytes (Na Cl and K) before "low") and after ("high") loading the glycogen depots in muscle tissue

Discussion

The influence of different diets on the size of the water retention in the organism has been discussed during the last 100 years (Pavy 1860, Benedict and Milner 1907) Several authors have stated that carbohydrate diet causes the largest water retention (Puckett and Wiley 1932, MacKay and Bergman 1932) It was also demonstrated early that a carbohydrate diet caused storage of glycogen in the liver and it was confirmed that to liver glycogen water was bound at the storage (Pavy 1860, Bridge and Bridges 1932)

In the present study an increase in glycogen content of the muscle with 15 4 g/kg wet muscle was accompanied by an increase in body weight by 2 4 kg and in total body water by 2 2 l

Even if the change in muscle glycogen in the thigh and the arm is considered as representative of changes in the whole muscle mass, it is impossible to exactly calculate the changes in total glycogen content of the body without knowing the size of the muscle mass Using indirect calorimetri during prolonged exercise Hedman (1957) estimated the normal total glycogen content of the body to around 400 g A division of this amount by the mean glycogen concentration found in muscle tissue (Hultman 1967) gives a muscle mass of 30 kg for Hedman's subjects This value agrees well with the fact that around 40 per cent of the body weight consist of muscles in a normal healthy man

Total potassium determination is another possible way to approximately measure the muscle mass of the body (Talso *et al* 1960) In 10 of our 19 subjects the mean value for the total amount of potassium was 170 g (body weight 74 7 kg after mixed diet, body height 182 cm and skeletal weight 12 8 kg) The muscle mass then can be calculated to be approximately 35 kg (von Dobeln 1968) A mean change in glycogen content of 15 4 g/kg wet muscle then gives an approximate increase in the glycogen stores of 500 g There is no reason to believe that fat protein or skeleton tissue has increased or decreased during the period of carbohydrate diet This means that the rest of the increase in body weight by 1 9 kg may be water This value (1 9 l) agrees rather well within the increase of total body water observed in the whole material which was 2 2 l The mean error of the mehod used for determination of total body water volume of 40—50 l means that in a specific case the accuracy of the measurements cannot be better than 1 5—2 l This may explain the fact that in 3 of the 19 subjects studied the total body water had decreased somewhat (0 3—0 8 l) in spite of the glycogen storage It is worth noticing that in those 3 cases (subjects 3 4 and 13) increase in glycogen as well as in body weight was relatively small and less than the mean for the group

In the present study there is a great uncertainty in calculating the volume of water that each gram of glycogen possibly binds at storage But if in the present study an increase of glycogen storage of 500 g is accepted it will be found that 3—4 g of water per gram of glycogen have been stored at the deposit of carbohydrate in the body

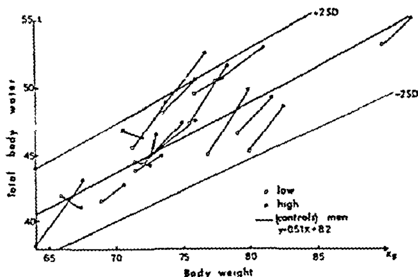


FIG 4 Total body water and body weight. The regression line from a control material of 3? men is given ± 2 × S D (error of method) Total body water is determined before ("low") and after ("high") loading the glycogen depots in muscle tissues of the examined students

How and where water is stored cannot be told on the basis of the present studies. Fenn (1939) showed that the extracellular volume measured as "the chloride space" did not increase parallelly to the increase of glycogen stored in the liver and he suggested that at least the main part of the water was stored with glycogen intra cellularly. Sreter showed in 1963 with inulin as tracer substance that the "extra-cellular space" increased in muscle tissue after work and the decrease of the "intra-cellular space" might be caused by the water released by the reduction of glycogen. Kozlowski and Saltin (1964) got to the same conclusion on basis of measurements of "thiosulphate space" and plasma volume before and after dehydration caused by hard physical work. *Of special interest in that study was the fact that after dehydration* caused by high room temperature but no physical exercise the plasma volume and "extra-cellular space" decreased significantly more than after a corresponding degree of dehydration during physical work. In a material of muscle biopsies in humans it has been shown that the intra-cellular volume of water decreases with a reduction in glycogen content (Ahlborg *et al* 1967)

One important conception before making any conclusions about the total water data in the present study is that the subjects were not hypo- or hyperhydrated at any occasion when total body water measurements were done. The subjects were young and healthy and had normal kidney function. During the whole experiment water was given in excess to the sensation of thirst which was controlled by determination of "total water exchange" from "turn over rate of water" (Olsson 1970). The determinations of urine volumes, specific weight of urine and the potassium/sodium ratio in urine did not at any occasion show values indicating retention of

fluid caused by too low volume of body water The volume of urine decreased, however, during the last two days of the carbohydrate diet period parallelly with an increasing storage of glycogen in the muscles, which in fact speaks for water retention as there was no change in the subjects' usual activity and the same volume of water was given Serum electrolytes and plasma volumes were also unchanged in situations "low" and "high" The present results seem then to demonstrate that a change in the muscle glycogen content of the body causes the body weight to increase significantly more than the amount of stored glycogen

Further this extra increase in weight is due to deposition of water, which probably takes place in the cells In Fig 4 the total body water is related to the body weight with "low" and "high" muscle glycogen content With the lower muscle glycogen content the ratio between total body water and body weight is $0\,622 \pm 0\,03$ compared with $0\,631 \pm 0\,04$ in the situation with a high muscle glycogen content As the difference is significant ($p < 0\,05$) it means that the body contains relatively more water when the glycogen is stored in the body The total amount of glycogen which can be stored in the body gives an increase in total body water which is larger than the error of the method It may therefore be of importance to take the glycogen content of the body into consideration when total body water is determined

We are grateful for the help from dr W von Dobeln who carried out the determinations of total potassium content We also acknowledge the help from dr Stig Johansson Clinical Department Karolinska sjukhuset who has carried out the chemical analyses of blood

This study has been supported by grants from the Swedish Medical Research Council (projects No K67 14X 2202 01 and No B26X 740 01) and Carl Bertel Nathhorsts vetenskapliga och allmannyttiga stiftelser

References

AHLBORG B J BERGSTROM L G EKELUND and E HULTMAN Muscle glycogen and muscle 1967 *70* 129—142

BE of water and energy in *175* 224—226

B t muscle glycogen and physical performance *Acta physiol scand* 1967 *71* 140—150

BRIDGE E M and E M BRIDGES The relation of glycogen to water storage in the liver *J biol Chem* 1932 *96* 381—386

DOBELN W VON Skelet en lichaamssamenstelling De samenstelling van het menselijk lichaam *Antwerpen Standard Wetenschappelijke Uitgeverij* 1968 93—99

FENN W O The deposition of potassium and phosphate with glycogen in rat livers *J biol Chem* 1939 *128* 297—307

HALEY H B I S EDELMAN and F D MOORE The turn over rate of body water in the human *Surg Forum* 1951 595—601

HEDMAN R The available glycogen in man and the connect on between rate of oxygen intake and carbohydrate usage *Acta physiol scand* 1957 *40* 305—321

HULTMAN E Muscle glycogen in man determined in needle biopsy specimens *Scand J clin Lab Invest* 1967 *19* 209—217

KOZLOWSKI, S and B SALTIN Effect of sweat loss on body fluids *J appl Physiol* 1964 *19* 1119—1124

LEHMANN I Elektrometrische Mikrobestimmung von Chlor in Vollblut Serum und Harn *Acta paediat* 1939 *26* 258—267

MACKAY E M and M C BERGMAN The relation between glycogen and water storage in the liver *J biol Chem* 1932 *96* 373—380

OLSSON, K. E., Determination of total body water and its turn over rate. A methodological study with tritium labeled water. *Acta chir. scand.* 1970. In press.

[illegible] n water in the total body water [illegible] 1970. In press.

[illegible] to water storage in the liver. *J* [illegible]

ROBERTS, J. E., K. D. FISHER and T. H. ALLEN, Tracer methods for estimating total water [illegible]

[illegible]

TALSO, P. J., C. E. MILLER, A. J. CARBALLO and S. VASQUEZ, Exchangeable potassium as a parameter of body composition. *Metabolism* 1960. 9. 456—471.

Acta physiol. scand. 1970. 80. 19—28

From the Department of Experimental Ophthalmology, the Wallenberg Laboratory, Institute of Pharmacology, University of Uppsala Sweden

Blood Flow and Oxygen Extraction in the Cat Uvea at Normal and High Intraocular Pressures

By

ALBERT ALM AND ANDERS BILL

Received 12 January 1970

Abstract

ALM A and A BILL *Blood flow and oxygen extraction in the cat uvea at normal and high intraocular pressures* Acta physiol scand 1970 *80* *19—28*

The rate of blood flow through the uvea and the arterio venous O_2 difference across the uveal tract were determined in cats The normal rate of blood flow was 1 14±0 23 ml/min and the normal a v O_2 difference was 1 02±0 16 volume per cent The O_2 extraction was 8 0±0 6 μl NTP/min The arterial O_2 saturation P_{O_2} P_{CO_2}, and pH were 95 6±0 3 per cent, 93±3 mm Hg 25 8±1 0 mm Hg and 7 44±0 01 units, respectively An artificial rise in eye pressure reduced the rate of uveal blood flow and increased the a v O_2 difference The O_2 extraction was relatively constant at blood flow rates above 0 3—0 5 ml/min At lower flow rates the O_2 extraction decreased in spite of a high venous O_2 concentration Blood collected from the [illegible]

[illegible]

some tissue supplied by the uveal vessels in spite of a relatively well oxygenated venous blood

In cats as in humans the blood flow through the uvea is of importance for the nutrition of the uvea itself the outer part of the retina, and the avascular tissues of the eye Previous studies have shown that the blood flow through the uvea is very high (Bill 1962 a) and that it is a function of the perfusion pressure, defined as the mean arterial blood pressure (MAP) minus the intraocular pressure (IOP) Each reduction in the perfusion pressure is accompanied by a reduction in uveal blood flow (Bill 1962 b and 1967)

Several investigators have found that blood collected from veins draining parts of the uvea has a high O_2 content, the a-v O_2 difference being less than 1 0 volume per cent (Cohan and Cohan 1963 a, Elgin 1964 and Pilkerton, Bulle and O'Rourke 1964) Cohan and Cohan (1963 b) reported that reductions in uveal blood flow, caused by increasing doses of pentobarbital sodium, had no detectable effect on the a v O_2 difference across the uveal tract If this applied also when the perfusion pressure is altered by changes in the IOP it would mean that even a moderate increase in the IOP above the normal level reduces the oxygen supply to the uveal tissue

OLSSON, K. E., Determination of total body water and its turn over rate. A methodological study with tritium labeled water. *Acta chir. scand.* 1970. In press.
OLSSON, K. E. and L. O. PLANTIN, The distribution of tritium water in the total body water. *An experimental study on man and rabbit. Acta chir. scand.* 1970. *In press.*
PAVY, F. W. *Phil. Tr. Roy. Soc.*, London 1860. *150*. 595.
PUCKETT, H. L. and F. H. WILEY, The relation of glycogen to water storage in the liver. *J. biol. Chem.* 1932. *96*. 367—371.
ROBERTS, J. E., K. D. FISHER and T. H. ALLEN. Tracer methods for estimating total water exchange in man. *Phys. in Med. Biol.* 1958. *3*. 7—15.
SNEDECOR, G. W., *Statistical methods*, The Iowa State College Press. U.S.A. 5th Ed. 1956.
SRETER, F. A. Distribution of water, sodium and potassium in resting and stimulated mammalian muscle. *Canad. J. Biochem.* 1963. *41*. 1035—1045.
TALSO, P. J., C. E. MILLER, A. J. CARBALLO and S. VASQUEZ, Exchangeable potassium as a parameter of body composition. *Metabolism* 1960. *9*. 456—471.

B Oxygen extraction from uveal blood at different eye pressures II

method (Beckman spectrophotometer DB-G, Beckman Instruments Inc, Fullerton, Cal) (See Holmgren and Pernow 1959) The mean of 2—4 determinations differing 2 % O_2 saturation or less was accepted

In this series polarographic determinations of the O_2 tension in arterial and venous blood were made with a modified Clark electrode (Beckman Physiological Gas Analyzer, model 160 Beckman Instruments, Inc, Palo Alto, Cal) The electrode was calibrated with two water-saturated gas … b/ 10 1 % O_2 and 6 3 % CO_2 in N_2 … (AGA, Lidingö, Sweden) A blood gas … 1966) was determined by measuring th… indrical glass tonometer at 38° C with two different water saturated gases of known O_2 tension Appropriate corrections were made for this factor, 1 029 as well as for temperature differences between the animal and the water bath of the O_2 electrode (se Severinghaus 1965)

The analytical error … Hilts and Karendal … glass syringes contai… were made within 10 … were $+0\ 1 \pm 1\ 9$ mm Hg

… with glass electrodes from … co_2-electrode was checked … CO_2 The gas had passed … differ more than 2 … with a buffer, pH … the same sample … perature between th…

C The effect of hemorrhage on oxygen saturation in the venous blood from the choroid and the anterior uvea

In this series only one needle was shot through the cornea for IOP measurements and no …

that it could be expected to collect blood largely from the anterior uvea (Fig 1) (see Bill 1963) The perfusion pressure was lowered by bleeding the animals The analyses of the O_2 saturation of arterial and venous blood were made with the same oximeter as used in series A

Results

In series A and B the uveal blood flow was determined in 11 expts in all In some of the experiments the ASCV or the tubing draining the vein was too narrow to take over the drainage of all uveal blood without a rise in the IOP In the experiments the postcannulation IOP was thus abnormally high In these experiments in which the postcannulation IOP was within the limits 10—30 cm H_2O the uveal blood flow was $1\ 14 \pm 0\ 23$ ml/min ($n = 5$) [1]

In the experiments where anesthesia was maintained with pentobarbital sodium the MAP was 167 ± 11 cm H_2O, and in those where it was maintained with uretan and chloralose the MAP was 160 ± 16 cm H_2O

[1] Here and in the following mean and S E are given

Fig 1 Frontal view of a section of the eye Two [illegible] one is located in plexus (A S C V = anterior superior ciliary vein)

In 7 expts the eye not used in the experiments was enucleated and dissected The uvea was weighed *to make it possible to estimate approximately the O_2 consumption* per 100 g tissue per min In 5 animals the lens was weighed and in 5 animals the retina was weighed The weights of the uvea, the lens, and the retina were 275 ± 5 mg, 697 ± 27 mg, and 90 ± 5 mg respectively

The IOP was raised in steps of 15—20 cm H_2O to a level between 85 and 150 cm H_2O Fig 2 shows the relationship between the uveal blood flow and the perfusion pressure Each point of the curve is the mean of 1—6 determinations as a rule 2 or 3 The figure at the right end of each curve represents the postcannulation IOP

[illegible] e perfusion pressure The changes in the IOP The postcannulation IOP [illegible] unfilled symbols belong to series A

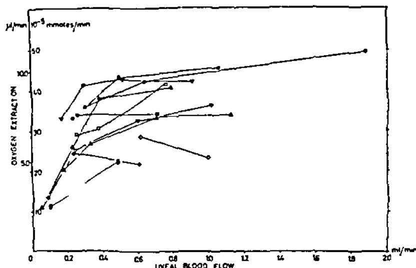


Fig 3 The oxygen extraction at different levels of uveal blood flow in series A and B The values for oxygen extraction in μl/min are calculated at NTP The same symbols are used for the different experiments as in Fig 2

Oxygen extraction from uveal blood at different rates of blood flow

The a v O_2 difference at postcannulation IOP levels of 10 to 30 cm H_2O was 1 02 ± 0 16 volume per cent (n=5) This represents 7 4 ± 1 2 per cent of the available O The O extraction in all the 11 expts at postcannulation IOP (including those with levels above 30 cm H_2O) was 8 0 ± 0 6 μl NTP/min or 35 6 ± 2 9 × 10^{-5} mmoles/min

Fig 3 shows the O_2 extraction at different rates of blood flow in the 11 expts As in Fig 2 each point represents the mean of 1—6 determinations When the uveal blood flow is reduced there is only a small reduction in the oxygen extraction at levels *of blood flow above 0 3—0 5 ml/min Further reductions in the uveal blood flow* result in a manifest reduction in the extraction of oxygen

The oxygen saturation, P_{O_2}, P_{CO_2}, and pH of arterial and venous blood

The arterial O_2 saturation was determined in all experiments, the P_{O_2} and P_{CO_2}, and pH in arterial blood in series B only The mean values for each animal was calculated from 3—5 determinations at different *times* The *mean and S E of the mean* values were O saturation 95 6 ± 0 3 % (n=15) P_{O_2} 93 ± 3 mm Hg (n=6) P_{CO_2} 25 8 ± 1 0 mm Hg (n=5) and pH 7 44 ± 0 01 units (n=6) The arterial P_{CO_2} is lower than the corresponding value in humans, but similar to that reported *for awake unrestrained cats (Fink and Schoolman 1962 Sorensen 1967* and Blosch 1969)

Fig 4 shows the a v differences at different levels of uveal blood expts are presented since in one of the ments the P_{CO_2} electrode

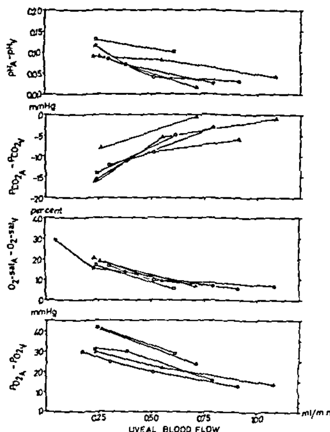


Fig 4 The arterio venous differences for oxygen saturation, P_{O_2}, P_{CO_2}, *and pH at different rates of uveal blood flow in series B The same symbol in the different figures represents the same experiment*

Measurements of the P_{O_2}, P_{CO_2}, and pH in venous blood offered certain difficulties due to the sampling circumstances The blood had to pass through a thin polyethylene tubing before it could be collected under paraffin oil in an ice bath At low levels of uveal blood flow this passage was slow To estimate the effects on the blood gases of this passage blood samples were passed through a drawn polyethylene tubing of the same diameter and length as used in the experiments Measurements before and after the passage at a flow rate of 0 2 ml/min failed however, to show any changes in the P_{O_2} or pH The P_{CO_2} was reduced with at the most 3 mm Hg at high levels of P_{CO_2}

Another difficulty was the effect of the dilution of the venous blood with 0 9 % saline used to perfuse the anterior chamber This dilution was pronounced only at high levels of IOP *In all experiments the dilution was less than 10 per cent at IOP* levels lower than 55 cm H_2O At the highest IOP the "blood" collected was a 1 1 mixture of blood and saline

Measurements of diluted and undiluted blood samples with a P_{O_2} of 70—75 mm Hg, P_{CO_2} of 50—55 mm Hg, and pH of 7 40—7 50 units were made to estimate the changes due to the dilution The saline was saturated with room air No changes

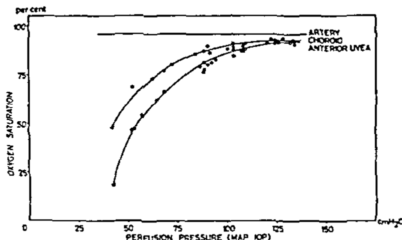


Fig 5 The arterio-venous differences for oxygen saturation at different perfusion pressures in one of the experiments in series C. Black dots represent mainly choroidal blood and unfilled dots represent blood collected largely from the anterior uvea

could be detected in the P_{O_2} though blood and saline were mixed in equal parts The P_{CO_2} on the other hand showed a reduction almost proportional to the dilution Small changes in the pH were detected with a maximum of 0 02 units when blood with pH of 7 2—7 4 and saline were mixed in equal parts The values shown in Fig 4 have been corrected as for the dilution effect on P_{CO}

Oxygen saturation in blood from the choroid and from the anterior uvea at different perfusion pressures

The O_2 saturation was determined in venous blood collected mainly from the choroid and in blood presumed to come largely from the anterior uvea Choroidal venous blood showed a higher O_2 saturation than blood from the anterior uvea a difference that was more pronounced at low perfusion pressures than at high pressures In blood from both regions the O_2 saturation decreased with reductions in the perfusion pressure with no corresponding reductions in the arterial O_2 saturation. Fig 5 shows the result of one of the 4 expts The 3 others gave similar results

Discussion

The normal figures for IOP and MAP in cats during general anesthesia are 20—30 cm H_2O and 150—170 cm H_2O respectively (see Bill 1963 and 1964 and Eakins 1969) Thus the perfusion pressure defined as in the introduction normally is 120—150 cm H_2O The true perfusion pressure however is about 25 cm H_2O lower due to a fall in blood pressure in the ophthalmic and ciliary arteries (Bill 1967)

The results of the experiments reported here confirm the previous observation that at normal IOP and MAP the blood flow through the cat uvea is very high and that it is reduced by artificial increments in IOP (Bill 1962)

As pointed out previously, in some of the experiments the postcannulation IOP was above the normal range, which gave a somewhat low initial blood flow Still these experiments could be used to determine at what level of blood flow the O_2 extraction is reduced since this reduction was observed only at very low flow rates

In those of the present experiments where the IOP was 10—30 cm H_2O the a v O_2 difference was very small The average was 1 02 volume per cent, a figure that is comparable with that in the kidney (Kramer and Deetjen 1960 and Leichtweiss *et al* 1969)

As mentioned the uvea the lens and the outer part of the retina (Collier 1967) are nutriated by the blood flow through the uvea There is no reason to except great losses of O_2 from the uveal blood to the sclera and the cornea takes most of its O_2 from the air (Langham 1952 and Fatt and Bieber 1968) If the cat lens is assumed to consume O_2 at the same rate as the rabbit lens 47 4 µl per g tissue per hr (Field *et al* 1937) the approximative O_2 consumption of a cat lens weighing 700 mg is 0 6 µl per min. Thus the remaining 7 4 µl NTP O_2 per min probably corresponds to the O_2 consumption in the uvea and the outer part of the retina with a total weight of about 300 mg This will give an O_2 consumption in this mixed tissue of about 2 5 ml per 100 g tissue per min which is about two thirds of that in brain tissue, 3 3 ml per 100 g tissue per min (Kety and Schmidt 1948) In the brain different parts show marked differences in O_2 consumption (Dixon and Meyer 1936) One can expect similar or even more pronounced differences between the retina and the different parts of the uvea

Meesmann (1932 studied the effects of various drugs on ocular blood flow and O_2 consumption in the rabbit He noted great changes in outflow from an opened vortex vein after administration of Glaukosan but only small changes in O_2 consumption at the same time

Cohan and Cohan (1963 b) studied the a v O_2 difference across the uveal tract in dogs To obtain changes in the uveal blood flow they lowered the arterial blood pressure with large doses of pentobarbital sodium Although they succeeded in reducing the uveal blood flow to less than half of its initial value they found no change in the a v O_2 difference and therefore suggested that the O_2 extraction was proportional to the blood flow In our experiments reductions in uveal blood flow to one third of its normal value resulted only in small changes in O_2 extraction The reason for the difference between our results and those of Cohan and Cohan is not clear As they point out they did not collect all the blood leaving the uvea and had no assurance that their samples came from the same tissue regions at different flow rates Therefore a possible explanation is a redistribution in the blood flow through the eye with decreasing blood pressure Our experiments with blood samples collected from different parts of the uvea, however do no suggest any great difference between different areas neither in the venous O_2 saturation nor in the decrease in the O_2 saturation with

reductions in uveal blood flow. Another possibility is that large doses of pentobarbital sodium have an effect on the O_2 consumption in the retina and the uvea; it is known that the O_2 consumption is reduced in brain cells exposed to high concentrations of barbiturate (Wechsler, Dripps and Kety 1951, and Westfall 1951). We found no statistically significant difference in the O_2 extraction from uveal blood using different anesthetic agents. The figures were 8.3 ± 1.0 µl NTP/min and 7.7 ± 0.9 µl NTP/min for series A and B respectively.

The intraocular tissues seem to maintain the O_2 extraction at a relatively constant level in spite of reductions in uveal blood flow to one third of its normal value. Without doubt this capacity helps to protect the intraocular tissues against anoxia during attacks of acute glaucoma and during deep drops in blood pressure.

It is intriguing that in the experiments with stepwise reductions in uveal blood flow the O_2 extraction was clearly reduced at a flow rate at which the venous blood was still saturated with O_2 to 70—75 %. Several mechanisms may contribute to this phenomenon. Due to a high O_2 consumption in the basal layers of the retina these cells may need a high O_2 concentration in the choriocapillaries for adequate oxygenation. Exchange diffusion of O_2 between the arteriole and the small veins in the ciliary processes may tend to give a high O_2 saturation in venous blood in spite of a low O_2 concentration in the capillaries. It is also possible that parts of the uvea, e.g. some ciliary processes, loose all their blood supply at low perfusion pressures when there is still an adequate circulation in other parts of the uvea. Finally a-v anastomoses may play a role.

Supported by grant B70-14X-147-06C from the Swedish Medical Research Council and by grant EY 00475 from the National Eye Institute, US Public Health Service.
Thanks are due to Miss Anita Persson and Miss Monica Thoren for valuable technical assistance.

References

BARANY, E. H.: The mode of action of pilocarpine on outflow resistance in the eye of a primate (Cercopithecus ethiops). *Invest. Ophthal.* 1962, *1*, 712—727.
BARTELS, H. and H. HARMS: Sauerstoffdissoziationskurven des Blutes von Saugetieren. *Pflugers Arch. ges. Physiol.* 1959, *268*, 334—365.
BILL, A.: A method for quantitative determination of the blood flow through the cat uvea. *Arch. Ophthal.* 1962a, *67*, 156—162.
BILL, A.: Intraocular pressure and blood flow through the uvea. *Arch. Ophthal.* 1962b, *67*, 336—348.
BILL, A.: The uveal venous pressure. *Arch. Ophthal.* 1963, *69*, 780—782.
BILL, A.: The intrascleral venous pressure in the eye of the cat. *Exp. Eye Res.* 1964, *3*, 188—191.
BILL, A.: Uveal circulation: a review of methods and results. *Glaucoma Symp. Tutzing Castle* (1967), pp. 52—72 (Karger, Basel/New York)).
BLOSCH, M.: Das Saure-Basengleichgewicht der Katze und seine Veranderungen durch die Narkose und kunstliche Beatmung im akuten neurophysiologischen Experiment. *Z. Biol.* 1969, *116*, 314—320.
COHAN, B. E. and S. B. COHAN: Flow and oxygen saturation of blood in the anterior ciliary vein of the dog eye. *Amer. J. Physiol.* 1963a, *205*, 60—66.
COHAN, B. E. and S. B. COHAN: Anterior ciliary venous blood flow and oxygen saturation at reduced arterial pressures. *Amer. J. Physiol.* 1963b, *105*, 67—70.
COLLIER, R. H.: Experimental embolic ischemia of the choroid. *Arch. Ophthal.* 1967, *77*, —692.

DIXON T F and A MEYER Respiration of brain *Biochem J* 1936 *30* 1577—1582
EAKINS K E A comparative study of intraocular pressure and gross outflow facility in the cat eye during anesthesia *Exp Eye Res* 1969 *8* 106—115
ELGIN S S Arterio-venous oxygen difference across the uveal tract of the dog eye *Invest Ophthal* 1964 *3* 417—426
FATT I and M T BIEBER The steady state distribution of oxygen and carbon dioxide in the in vivo cornea I The open eye in air and the closed eye *Exp Eye Res* 1968 *7* 103—112
FIELD J JR E G TAINTER A W MARTIN and H S BELDING Studies on the oxygen consumption of the rabbit lens and the effect of 2—4 dinitrophenol thereon *Amer J Ophthal* 1937 *20* 779—794
FINK B R and M SCHOOLMAN Arterial blood acid base balance in unrestrained waking cats *Fed Proc* 1962 *21* 440
HILTY H and B KARENDAL Effect of syringe material on oxygen tension in stored blood *Acta Soc Med upsalien* 1969 *74* 193—203
HOLMGREN A and B PERNOW Spectrophotometric measurements of oxygen saturation of blood in the determination of cardiac output A comparison with the Van Slyke method *Scand J clin Lab Invest* 1959 *11* 143—149
KETY S S and C F SCHMIDT The nitrous oxide method for the quantitative determination of cerebral blood flow in man Theory procedure and normal values *J clin Invest* 1948 *27* 476—483
KRAMER K and P DEETJEN Beziehungen des O Verbrauchs der Niere zu Durchblutung und Glomerulusfiltrat bei Änderang des arteriellen Druckes *Pflugers Arch ges Physiol* 1960 *271* 782—796
LANGHAM M Utilization of oxygen by the component layers of the living cornea *J Physiol* (Lond) 1952 *117* 461—470
LEICHTWEISS H P D W LUBBERS CH WEISS H BAUMGARTL and W RESCHKE The oxygen supply of the rat kidney measurements of intrarenal pO_2 *Pflugers Arch ges Physiol* 1969 *309* 328—349
MEESMANN A Weitere Untersuchungen uber die Blutumlaufzeit und den Sauerstoffverbrauch des Kaninchenauges *Ber Dtsch ophthal Ges* 1932 *49* 268—277
MORAN F L J KETTEL and D W CUGELL Measurements of blood pO with the micro cathode electrode *J appl Physiol* 1966 *21* 725—728
PILKERTON R P H BULLE and J O ROURKE Uveal tissue respiration and glycolysis in living experimental animals *Invest Ophthal* 1964 *3* 237—240
SEVERINGHAUS J W Blood gas concentrations *In Handbook of Physiology, section 3 Vol II Respiration* 1965 1475—1487
SORENSEN S C Arterial pCO_2 in awake cats calculated from gas tensions in subcutaneous pockets *Resp Physiol* 1967 *3* 261—265
WECHSLER R L R D DRIPPS and S S KETY Blood flow and oxygen consumption of the human brain during anesthesia produced by thiopental *Anesthesiology* 1951 *12* 308—314
WESTFALL B A Effects of pentobarbital amobarbital and barbital on oxygen consumption of brain slices *J Pharmacol xp Ther* 1951 *101* 163—166

Acta physiol. scand. 1970 80. 29—38

From the First Medical Service Sahlgrenska Sjukhuset University of Goteborg Sweden

Effects of Feeding States on Lipid Radioactivity in Liver, Muscle and Adipose Tissue after Injection of Labelled Glucose in the Rat

By

P BJORNTORP, M KROTKIEWSKI, B LARSSON AND Z SOMLO-SZUCS

Received 16 January 1970

Abstract

BJORNTORP, P, M KROTKIEWSKI, B LARSSON and Z SOMLO-SZUCS *Effects of feeding states on lipid radioactivity in liver, muscle and adipose tissue after injection of labelled glucose in the rat* Acta physiol scand 1970 *80* 29—38

[illegible]

was most pronounced in the refed rats Adipose tissue showed higher fatty acid synthesizing activity than the liver in ad libitum fed but not in refed rats Glyceride-glycerol radioactivity was rather high in muscle In adipose tissue it was more constant in different feeding states than fatty acid radioactivity was By additions of measured radioactive metabolites glucose uptake in adipose tissue was roughly estimated to a few per cent of total glucose uptake

Recent findings of hyperinsulinemia in obesity (Karam Grodsky and Forsham 1963) and hyperlipemia (Nikkila *et al* 1965) indicate a block for glucose uptake in tissues where this uptake is facilitated by insulin The location of such a block is not known Even information on the quantitative role of different tissues as acceptors of glucose under physiological conditions is sparse probably due to the limited and complicated approaches to the problem

Adipose tissue has recently been suggested to be at least one site for insulin resistance in obesity (Salans *et al* 1968) The significance of such a resistance in adipose tissue for the decrease in glucose disappearance rate from plasma after a glucose load (decreased glucose tolerance) and the increased insulin production often seen in obesity is however not known because the uptake of glucose in adipose tissue in relation to total tissue uptake is not known. In vitro data from human adi

tissue indicate a small uptake of glucose in total adipose tissue (Bjorntorp *et al* 1968) If this is the case also in vivo an insulin resistance in adipose tissue would be expected probably to be of minor importance for the production of a decreased glucose tolerance and a compensatory hyperinsulineamia in obesity

In order to try to elucidate this question the transformation of glucose into lipids in the rat under different physiological feeding conditions in three central tissues for carbohydrate and lipid metabolism was measured, *viz* liver, muscle and adipose tissue In preparatory experiments labelled glucose was injected and measurements performed in blood to try to define an early time where recirculation of label was not yet higher than allowing interpretation of tissue label of lipids

Material and methods

Administration of radioactivity

Male, 250—285 g Sprague Dawley rats were used One group of rats were fed ad libitum of a commercial rat food (EWOS Sodertalje, Sweden), containing 22 5 % protein 5 0 % fat

[illegible]

Corp Langen, Germany, NEC-042 P batch 368 30 1) in 0 5 g per kg b w of non labelled glucose in a total volume of 1—1 5 ml of water, followed by a small volume of normal saline Blood samples were taken for glucose determinations (Levin and Linde 1962) from the tail vein before glucose injection 45 min after glucose injection, and at 60 min, when the rat was sacrificed another blood sample was obtained from the carotid vessels

Tissue sampling

After 60 min the rats were decapitated, and the following tissues immediately removed and weighed Liver 2—4 g of muscles from the back epididymal fat pads divided into one proximal thick and one distal thin part omental fat and perirenal fat These samples (only 1—2 g of the liver) were then homogenized in 5 ml of 0 25 M sucrose in a beaker with an Ultra Turrax (Janke & Kunkel K G, Staufen i Br Germany) One ml of the homogenate was extracted in 15 ml chloroform methanol 2 1 (v v), filtered into a 25 ml measuring cylinder filter washed with 2 portions of each 2 ml chloroform methanol and then finally 5 ml of 0 05 M acetic acid were added After shaking the cylinder was left over night, and aliquot parts of the chloroform phase evaporated in counting vials and counted in 10 ml of 0 4 % 2 5-diphenyloxazole and 0 01 % p-bis-2 (5 phenyloxazolyl) benzene (Packard La Grange Illinois USA in toluene Another chloroform aliquot was evaporated to dryness an excess of KOH in ethanol added and saponification performed at 60° C during one hour After acidification extraction with heptane was performed Radioactivity in this fraction was then determined as with total lipid radioactivity It was analysed with thin layer chromatography in some samples and found to reside in fatty acids in agreement with a report of low cholesterol synthesis from glucose as cor [illegible] the three tissues studied Janser [illegible] radioactivity and fatty acid [illegible] sumably contained glycerol [illegible] lipids

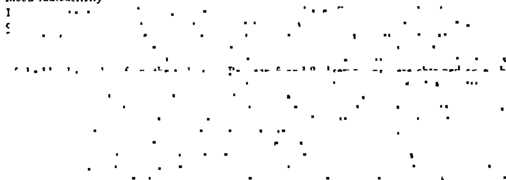
Quenching when present was corrected for by the internal standard technique using ^{14}C-toluene or U ^{14}C-glucose as standard

The purity of the radioactive glucose used was checked in the following way Only traces of radioactivity were extracted from a water solution into heptane Ninetyeight per cent of the radioactivity was found in one spot corresponding to the Rf of glucose on thin layer chromatograms (Bjorntorp 1966

Determination of muscle and adipose tissue mass

Total muscle mass was determined by the following procedure From eviscerated rats skin feet tail and head were removed and the remaining carcass was placed in 300 ml concentrated nitrous acid and left until dissolved After measuring of the total volume of this solution an

aliqout was taken for determination of potassium contents (Lindholm 1967) The total potas-



The heptane phase was treated with dilute potassium hydroxide as described by Havel, Naimark and Borchgrevink (1963) to obtain free fatty acids as potassium soaps in an ethanol phase Radioactivity in the heptane was then counted directly and after saponification to give radioactivity in total lipid soluble material after saponification and acidification (mainly fatty acids and cholesterol) After acidification of the ethanol phase, the plasma free fatty acids were extracted into heptane and counted

Results

Fig 1 shows the results of the determinations in blood at different times in *ad libitum* fed rats It may be seen that blood glucose concentration had returned approximately to the preinjection values at 45 min Glucose specific activity was not different at 25 and 45 min while it was lower at 60 and 85 min than at 45 min ($p<0.02$ respectively <0.001) Small amounts of radioactivity appeared in the heptane phases of the extracts at 45, 60 and 85 min but these counts were too few to be possible to quantitize accurately Measurements in larger blood samples at 60 min (obtained from the carotid vessels at decapitation) gave values of less than 0 004 % of total injected radioactivity per ml blood in the heptane extracts None of this radioactivity was found in the fatty acids

Table I gives the original data of the measured amounts of radioactivity per gram of tissues These data were then utilized for different calculations in the following presentation Table I also gives blood glucose values and weight of rats in these experiments

Table II shows the results of distribution of radioactivity in the whole liver and in estimated total muscle and adipose tissue in total lipids and in glyceride fatty acids and glycerol

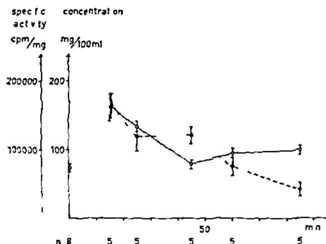


Fig 1 Blood glucose concentration (mg/100 ml whole line) and spe cific activity (CPM/mg glucose dashed line) in rats at different times after injection of 2 μc (U ^{14}C) glucose and 0 5 g non labelled glucose per kg body weight i v Means ±SEM n = number of rats

TABLE I Distribution of i v injected radioactivity from (U ^{14}C) glucose per gram of different tissues in the rat 60 min after injection

	Ad libitum fed rats		*Fasting rats*		*Refed rats*	
	C M	*SAP*	*C M*	*S 1P*	*C M*	*S 1P*
Liver	0 04 ±0 001 12	0 01 0 002 (12	0 03 +0 003 (9)	0 (9)	0 12 ±0 01 (10)	0 05 ±0 003 (10)
Muscle	0 02 ±0 03 (12	0 004 ±0 002* (5	0 008 ±0 001* (9)	0 (9)	0 02 ±0 004 (10)	0 002 ±0 001 (10)
Proximal fat pad	0 04 −0 01 (13	0 01 ±0 003 (13	0 02 ±0 02 (9)	0 (9)	0 04 ±0 003 (10)	0 02 ±0 005 (10)
Distal fat pad	0 09 0 03 (13	0 02 0 006 13	0 02 ±0 005 (9)	0 (9)	0 12 ±0 01 (10)	0 05 ±0 005 (9)
Omental fat	0 10 0 02 13	0 03 +0 003 12	0 02 ±0 0001 9)	0 (8)	0 13 ±0 02 (10)	0 05 ±0 006 (10)
Perirenal fat	0 12 0 01 13	0 04 ±0 004 11	0 05 +0 0001 (9)	0 005 +0 001 (8)	0 20 ±0 03 (10)	0 03 ±0 01 (8)
Weights of rats g	256 8 (5		250 ±11 (3)		303 +7 (10)	
Blood glucose 0 min	87 3 (13		61 ±11 (4)		95 ±3 (10)	
(mg/100 mg						
(45 min	95 5 13		81 +3 (4)		110 ±11 (9)	
(60 min	93 6 13		82 ±13 (4)		103 ±13 (4)	

* *counting error 10 2 5*

Rats were injected in the tail vein with 2 μC (U ^{14}C) glucose and 0 5 g/kg of non labelled glucose

within parenthesis are numbers of animals

TABLE II Distribution of lipid radioactivity from (U-^{14}C) glucose injected i v 60 min previously, in different tissues in the rat

	Muscle			Liver			Adipose tissue		
	Lipid	Fatty acid	Glycerol	Lipid	Fatty acid	Glycerol	Lipid	Fatty acid	Glycerol
Ad lib fed rats	1 61 ± 0 3 (12)	0 4 ±* 0 1 (5)	~1 21 ± 0 3 (5)	0 40 ± 0 03 (12)	0 10 ± 0 02 (12)	0 30 ± 0 03 (12)	0 90 ± 0 10 (13)	0 38 ± 0 05 (11)	0 52 ± 0 10 (11)
Fasting rats	0 7 ± 0 1 (9)	0 (9)	~0 7 ± 0 1 (9)	0 20 ± 0 04 (9)	0 (9)	0 20 ± 0 04 (9)	0 29 ± 0 11 (9)	0 01 ± 0 001 (8)	0 28 ± 0 10 (8)
Refed rats	1 41 ± 0 4 (10)	0 2 ±* 0 1 (10)	~1 2 ± 0 4 (10)	1 48 ± 0 19 (10)	0 60 ± 0 07 (10)	0 88 ± 0 18 (10)	1 29 ± 0 09 (10)	0 55 ± 0 08 (9)	0 74 ± 0 09 (9)

* counting error 10—25 %

In *ad libitum* fed rats muscle contained most lipid soluble label and liver the least After fasting lipid radioactivity was low in all three tissues, while in refed rats lipid soluble radioactivity was higher than in *ad libitum* fed rats both in adipose tissue ($p<0 01$) and liver ($p<0 001$)

The fatty acid data from muscle are determined with a rather low degree of precision. In *ad libitum* fed rats more radioactivity was found in adipose tissue as compared with liver ($p<0 001$) There were only traces of fatty acid radioactivity in adipose tissue after fasting In refed rats fatty acid synthesis was higher than in *ad libitum* fed rats in liver ($p<0 001$) and adipose tissue ($p<0 05$) and apparently not much different in these two tissues

Glyceride-glycerol radioactivity was determined with a low precision in muscle because of the low precision of the fatty acid determinations It seems, however, that this incorporation of glucose label was higher than in both liver and adipose tissue in *ad libitum fed and fasting rats In liver fasting rats* showed a lower value of borderline significance as compared with *ad libitum* fed rats ($p<0 10>0 05$) while refed rats incorporated more glucose radioactivity into glyceride-glycerol than both fasting ($p<0 01$) and *ad libitum* fed rats ($p<0 01$) In adipose tissue *ad libitum* fed and *fasted rats showed no significant difference while refed rats incorporated more glucose radioactivity into glyceride-glycerol than fasted rats* ($p<0 01$)

TABLE III Distribution of radioactivity from (U ^{14}C) glucose in the fatty acids per gram of different adipose tissues in the rat

	Ad libitum fed rats	*Fasting rats*	*Refed rats*
Proximal fat pad	0 011 ± 0 003 (13)	0 (9)	0 018 ± 0 005 (10)
Distal fat pad	0 021 ± 0 006 (13)	0 (9)	0 045 ± 0 005 (9)
Omental fat	0 030 ± 0 003 (12)	0 (9)	0 052 ± 0 006 (10)
Perirenal fat	0 042 0 004 (11)	0 005 ± 0 001 (8)	0 088 ± 0 011 (8)
Means ± SEM	0 035 ± 0 004	0 001 ± 0 00003	0 051 ± 0 007

Experimental details as in Table I and II and in the text Results are given as per cent of injected radioactivity
Means ± SEM Figures within parenthesis are number of animals

Table III gives the radioactivity per gram tissue in the fatty acids of adipose tissue from different regions It may be seen that fatty acid radioactivity was practically none after fasting while it was generally higher in the refed rats in comparison with the *ad libitum* fed rats The proximal fat pad contained less radioactivity than the distal in refed rats ($p < 0\ 001$) while perirenal fat contained more than epididymal fat tissues ($p < 0\ 01$ or lower) Perirenal adipose tissue from refed rats showed most radioactivity in fatty acids of all adipose tissue ($p < 0\ 02$ or lower)

Discussion

Recirculation of radioactivity

The finding of radioactivity in glyceride glycerol of blood lipids indicates that radio activity in lipids probably synthetized in the liver (Havel and Goldfien 1961), is recirculating This label is probably later taken up by adipose tissue and muscle (Bragdon and Gordon 1958) It was of a small magnitude and appeared shortly before measurements of radioactivity in tissues The complication for interpretations of tissue radioactivity caused by this recirculation is therefore probably limited No radioactivity was found in the fatty acids Therefore it seems likely that the measure ments of fatty acid radioactivity in different tissues should be true estimates of fatty acid synthesis in these tissues

The constant specific activity of glucose at 25 and 45 min after injection indicates that at this time injected glucose had equilibrated with plasma glucose and pre sumably extracellular glucose This assumption is also supported by the work of Shipley *et al* (1967) Sixty minutes after injection of radioactive glucose production of glucose from the liver had probably started, because the specific activity of blood glucose was then again decreasing (*cf* Fig 1)

Other water soluble radioactive metabolites were found in the bl of the sor

rats (20—35 % of blood glucose radioactivity) with an Rf value on the thin layer chromatograms corresponding to glycerol pyruvate lactate or β hydroxybutyrate Lactate pyruvate might have been produced in muscles during post mortem contractions (Baker and Huebotter 1964) while glycerol possibly might originate from hydrolysis of glyceride glycerol labelled triglycerides from the liver It seems plausible that most of this label is later taken up by the liver (Cori 1931) Pyruvate is however, a precursor to glyceride glycerol (Rose and Shapiro 1963) and glucose uptake in muscle and adipose tissue glyceride glycerol will therefore probably tend to be overestimated

Radioactivity in different regions of adipose tissue and muscle

Another difficulty for interpretation of results is the fact that it is impossible to measure the radioactivity in all the different regions of the diffusely spread out tissues of muscle and adipose tissue The data from adipose tissue from different regions give an impression of the magnitude of the difference in lipid synthesis in these regions As far as muscle is concerned no similar regional data were collected because the muscle piece investigated probably is reasonably representative, being a major part of a large, isolated muscle group containing different individual muscles

Lipid radioactivity in different tissues

In previous investigations on lipogenesis in vivo measurements have usually been performed a longer time after administration of label than in the present work and therefore incorporation of glucose label into total body fatty acids in these investigations is usually (Patkin and Masoro 1964 De Freitas and Depocas 1965 Masoro Chaikoff and Dauben 1949) but not always (Lequin and Steyn Parve 1962) higher Jansen Hutchison and Zanetti (1966) and Jansen Zanetti and Hutchison (1966) studying the early phase of glucose label incorporation into fatty acids also found a higher synthesis than in the present work when giving glucose perorally to mice in a much larger dose than that administered in the present work

Lipid radioactivity in muscle was high in all tested feeding conditions Most of this label resided in the glycerol moiety of the lipids The fatty acid radioactivity was small and of doubtful significance because the counting error was large Labelled precursors therefore probably were utilized for esterification of primarily non labelled fatty acids originating either from muscle lipids or from plasma lipoproteins

Liver radioactivity in glyceride glycerol is difficult to interpret because this metabolite had already begun to recirculate when measurements were performed as discussed above Liver radioactive fatty acids probably did not recirculate however because no radioactivity was discovered in blood fatty acids It appears that fatty acid synthesis in ad libitum fed rats was higher in adipose tisue than in liver while this was not the case in refed rats In fasting rats only traces of fatty acids were synthesized

In adipose tissue glyceride glycerol label was relatively constant in comparison with fatty acid synthesis which varied widely in different feeding states This in-

dicates a priority for glyceride glycerol synthesis in relation to fatty acid synthesis indicating that this conclusion from in vitro studies (Cahill *et al* 1960) is apparently valid also for in vivo conditions

In different adipose tissue regions there was a considerable variation in fatty acid synthesis as previously noted by Bray (1968)

Calculations of adipose tissue glucose uptake

The present results seem to allow approximate conclusions about total glucose uptake in adipose tissue in relation to other tissues By addition of labelled products of glucose in adipose tissue glucose uptake will be quantitized to the extent that recirculating radioactive metabolites of glucose will not invalidate such a calculation This recirculation (mainly pyruvate lactate) will probably rather tend to overestimate glucose uptake in adipose tissue as discussed above

Such a calculation would also require that the measured labelled metabolites in adipose tissue were so stable that they had not started to escape from adipose tissue when measurements were performed Labelled fatty acids were not found in blood therefore these probably were not pouring out of adipose tissue at the time of measurements Labelled glyceride glycerol was however found in blood It seems highly unlikely that it should originate from adipose tissue glyceride glycerol which then first had to go through the liver and into liver secretion of lipids Furthermore other investigations (Jansen Hutchison and Zanetti 1966) have indicated that adipose tissue glyceride glycerol should be stable for longer time than 60 minutes Labelled glyceride glycerol therefore probably had not been released from adipose tissue when measurements were performed The measured labelled metabolites of adipose tissue therefore probably had long enough half life to allow the quantitation in question

Glycogen synthesis in adipose tissue is limited (Vaughan 1961) Control experiments with extraction of adipose tissue homogenate in trichloroacetic acid did not show radioactivity above the amount expected from the contents in adipose tissue extracellular space if this was considered to contain the same specific activity of water soluble radioactivity as plasma at the time of sacrifizing animals (*cf* discussion below!) This indicates that uptake of glucose into water soluble metabolites in adipose tissue could be neglected for the present calculations

One end product of adipose tissue metabolism of glucose which can not be measured with the present technique is labelled carbon dioxide and this will have to be extrapolated from experiments in vitro where it has been found to be approximately half of total glucose uptake in *ad libitum* fed rats (Winegrad and Renold 1959, Cahill Leboeuf and Renold 1960)

With these approximations glucose uptake in total adipose tissue would be less than 2 % 0 5 % and 3 % of injected glucose in the *ad libitum* fed fasting respectively refed rats

The total glucose uptake in all tissues might also be approximated with the following assumptions The concentration of extracellular glucose radioactivity is

probably close to that of plasma when the rats were sacrifized as discussed above Total extracellular glucose radioactivity can then be estimated assuming an extracellular space of the rat of 20 % of the body weight (Moore *et al* 1963) When this is subtracted from injected radioactivity total glucose uptakes of 82 %, 79 % and 89 % are obtained for *ad libitum* fed, fasting and refed rats respectively This in turn gives adipose tissue uptake of glucose of less than 3 % of total tissue glucose uptake in *ad libitum* fed and refed rats In fasted rats it was less than 1 %

These considerations are of course valid only to the extent that the several assumptions utilized are valid These assumptions have, however, to be grossly erroneous to invalidate the conclusion that adipose tissue glucose uptake under the present conditions is small

The technical assistance of mrs Anita Hovden, mrs Agneta Eggers and miss Annika Ahlenius is gratefully acknowledged.

This work was supported by the Swedish Medical Research Council (grant No K 68-19 X 251 05) Dr Krotkiewski was supported by a grant from Forenade Liv and dr Somlo-Szucs by a grant from WHO

References

Baker N and R Huebotter, Glucose metabolism in mice *Amer J Physiol* 1964 *207* 1155—1160

Bjorntorp P The oxidation of fatty acids combined with albumin by isolated rat liver mitochondria *J biol Chem* 1966 *241* 1537—1543

Bjorntorp P T Schersten and A Gottfries Effects of glucose infusions on adipose tissue lipogenesis in man *Acta med scand* 1968 *183* 565—572

Bragdon J H and R S Gordon Jr Tissue distribution of C^{14} after the intravenous injection of labelled chylomicrons and unesterified fatty acids in the rat *J clin Invest* 1958 *37* 574—578

Bray G A A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Anal Biochem* 1960 *1* 279—285

Bray G A Lipogenesis from glucose and pyruvate in fat cells from genetically obese rats *J Lipid Res* 1968 *9* 681—686

Cahill G F Jr B Leboeuf and A E Renold Factors concerned with the regulation of fatty acid metabolism by adipose tissue *Amer J clin Nutr* 1960 *8* 733—739

Carlson L A Determination of serum glycerides *Acta Soc Med upsalien* 1959 *64* 208—213

Cori C F Mammalian carbohydrate metabolism *Physiol Re* 1931 *11* 143—275

Crofford O B and A E Renold Glucose uptake by incubated rat epididymal adipose tissue Rate limiting steps and site of insulin action *J biol Chem* 1965 *240* 14—21

De Freitas A S W and F Depocas Fatty acid and glyceride glycerol synthesis from glucose during high rates of glucose uptake in the intact rat *Canad J Biochem* 1965 *43* 437—450

Dole V P A relation between nonesterified fatty acids in plasma and the metabolism of glucose *J clin Invest* 1956 *35* 150—154

Havel R J and A Goldfien The role of the liver and of extrahepatic tissues in the transport and metabolism of fatty acids and triglycerides in the dogs *J Lipid Res* 1961 *2* 389—395

Havel R J A Naimark and C F Borchgrevink Turn-over rate and oxidation of free fatty acids of blood plasma in man during exercise Studies during continuous infusion of palmitate 1 ^{14}C *J clin Invest* 1963 *42* 1054—1063

Jansen G R C F Hutchison and M E Zanetti Studies on lipogenesis in vivo Effect of dietary fat or starvation on conversion of [^{14}C] glucose into fat and turnover of newly [illegible] 1966 99 [illegible]

vivo Effects of
1966 99 [illegible]

KARAM J H G M GRODSKY and P M FORSHAM Excessive insulin response to glucose in obese subjects as measured by immunochemical assay *Diabetes* 1963 *12* 197—204

LEQUIN H C and E P STEYN PARVE, Some aspects of glucose metabolism in normal and alloxan diabetic rats *Biochim biophys Acta* (Amst) 1962 58 439—448

LEVIN K and S LINDE, Determination of glucose in blood, cerebrospinal fluid and urine with a new glucose oxidase reagent *Svenska Lak Tidn* 1962 59 3016—3026

LINDHOLM B Body cell mass during long term cortisone treatment in asthmatic subjects *Acta endocr* (Kbh) 1967 *55* 202—221

MASORO E J I L CHAIKOFF and W G DAUBEN, Lipogenesis from glucose *in normal* and liverless animal as studied with C^{14} labelled glucose *J biol Chem* 1949 *179* 1117—1125

MOORE F D K H OLESEN, J D MURRAY H V PARKER M R BALL and C M BOYDEN *The body cell and its supporting environment* Philadelphia—London W B Saunders Co,

NIKKILA E A T A MIETTINEN M R VESENNE and R PEKONEN Plasma insulin in coronary heart-disease Response to oral and intravenous glucose and to tolbutamide *Lancet* 1965 2 508—511

PATKIN J K and E J MASORO Fatty acid synthesis in *normal* and cold acclimated rats *Canad J Physiol Pharmacol* 1964 *42* 101—107

ROSE G and B SHAPIRO Phosphoglyceric acid kinase activity and its role in adipose tissue *Bull R Coun Israel A* 1963 *11* 384—389

SALANS L B J L KNITTLE and J HIRSCH The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity *J clin Invest* 1968 *47* 153 165

SHIPLEY R A E B CHUDZIK A P GIBBONS K JONCEDYK and D O BRUMMOND Rate of glucose transformation in the rat by whole body analysis after glucose ^{14}C *Amer J Physiol* 1967 *213* 1149—1158

VAUGHAN M The metabolism of adipose tissue *in vitro* *J Lipid Res* 1961 2 293—316

WINEGRAD A J and A E RENOLD Studies on rat adipose tissue in vitro I Effects of insulin on the metabolism of glucose pyruvate and acetate *J biol Chem* 1958 *233* 267—272

Acta physiol. scand. 1970 80 39—44

From The Institute of Pharmacology University of Oslo Norway

Determination of Cortical Blood Flow in Rabbit Femur by Radioactive Microspheres

By

P K M LUNDE AND K MICHELSEN

Received 19 January 1970

Abstract

LUNDE, P K M and K MICHELSEN *Determination of cortical blood flow in rabbit femur by radioactive microspheres* Acta physiol scand 1970 *80* 39—44

The method for blood flow determination involving measurement of relative radioemission from radioactive microspheres retained in various tissues after intravascular injection has here been applied to minute tissue regions ^{85}Sr labelled plastic microspheres with a diameter of 15±5 μ were injected in a retrograde manner into the aorta in 10 anesthetized adult rabbits The tissues included in the present study were the m quadriceps femoris tendon the cortical bone and the corresponding bone marrow from the left femur diaphysis The unknown blood flows through these tissue regions were calculated by relating the measured radioactivity from tissue samples to a known blood flow and a known
posterior tibial artery The blood flo
tissue) Quadriceps femoris tendon —(
0 146 cortical bone of femur diaphysi

Due to the inaccessability of the tissue and the lack of suitable techniques our knowledge about blood flow magnitude in cortical bone is incomplete Increased knowledge about blood circulation in this particular tissue would be interesting from a physiological point of view and would also be of value in various surgical conditions

A technique involving the injection of radioactive microspheres into the circulation was introduced several years ago in cancer therapy (Ya *et al* 1961, Blanchard *et al* 1965) By using a small amount of such microspheres of a suitable size, usually about 50 μ in diameter retention of the spheres would take place in precapillary vessels of a corresponding diameter By measuring the emitted radioactivity from various tissue samples it has been shown that this activity corresponds well to the blood distribution between different tissues (Rudolph and Heymann 1967, Neutze, Wyler and Rudolph 1968) By correlating the emitted radioactivity from one region to the radioactivity from another region with a known flow, the flow in the former region can be calculated The advantage of this method is that it allows flow determination in minute otherwise inaccessable tissue regions

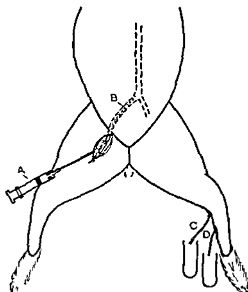


Fig 1 *Diagram of the experimental arrangement*

A Syringe for injection of radioactive microspheres into the rabbit's aorta via polyethylene catheter (B) in left femoral artery (for details see Fig 2) C and D Tubes for sampling of blood from two branches of the left posterior tibial artery

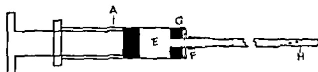


Fig 2 *Diagram with details of the injection syringe and the steel cannula in the tip of the polyethylene catheter*

A Injection syringe E Bolus with radioactive microspheres F Nylon filter with a mesh size of 20 μ G Rubber plug with hole for fixation of nylon filter H Steel cannula with radially placed bores with a diameter of about 100 μ

In the present study this method was applied for quantitative measurements of cortical bone blood flow in the femoral diaphysis of mature rabbits Besides the blood flow in cortical bone the blood flow in the femoral diaphyseal bone marrow and in the tendon of the quadriceps femoris muscle was determined

Methods

Animals and anesth s Mature rabbits of either sex weighing from 2 7 to 4 2 kg were used The animals were anesthetized by i v injection of 30—40 mg/kg b w of pentobarbitone (Nembutal® Abbott diluted 1 3 in 0 9 per cent NaCl) Heparin (750 i u /kg b w of pure powered heparin Novo was given i v to all animals just before the cannulation of the various vessels see below)

Preparation The main principles in the ex r nd 2 A polyethylene catheter was inserted in the artery so as to reach the abdominal aorta ju consisted of a 1 cm piece of a steel cannula placed bores in it each about 0 1 mm in diameter (Fig 2) This arrangement proved suitable to obtain an even intravascular distribution of the injected radioactive microspheres Two branches from the left posterior tibial artery were dissected free and cannulated for blood sampling The blood was allowed to run freely from the cannulas of these small vessels several minutes ahead of the injection of the radioactive microspheres to assure that a constant flow

i (3M
on was
stem

in rabbit tibia is about 15 μ. In the original batch about 1 g of microspheres, corresponding to 2.65 mC was suspended in 10 ml of 10 % dextran. Under the present conditions a total amount of microspheres corresponding to an activity of about 20 μC was sufficient for injec- [illegible]

size of 20 μ. Still there was a tendency for further formation of aggregates. Another nylon filter was therefore placed in the injection syringe (Fig. 2). Due to the rapid sedimentation rate of the microspheres the syringe had to be continuously shaken untill about 10 sec before the injection.

Blood sampling and injection procedure. Blood was collected continuously in one sample from each of the two tibial arteries for a period of 2—3 min. The bolus of radioactive microspheres was injected in the course of 3 to 5 sec into the abdominal aorta about 30 sec before the end of the sampling period. The animal was killed within 10 sec after the end of the sampling period by the i.v. injection of 300 mg of pentobarbitone (Nembutal® Abbott). The blood samples collected were weighed immediately for calculation of the blood flow through the two reference arteries. By comparing the radioactivity/flow ratios in these samples a possible skewness in distribution of the injected microspheres could be detected (see below).

Preparation of tissue samples. The tendon of the left quadriceps femoris muscle weighing about 0.2 g was isolated and sampled after removal of visible vessels and the fasciae. Then the left femur diaphysis was extirpated and the diaphysial part of the medulla was blown out. The osseous diaphysis was then crushed and all visible endosteal and periosteal structures were removed. All tissue samples were transferred directly into the tubes used in the scintillation detector and their wet weight was determined.

The blood and tissue samples were counted for ^{85}Sr activity in a 3 channel Auto-Gamma Spectrometer, (Model 5022 Packard Instrument Comp.).

Evaluation of the blood flow circulation. As mentioned above two reference arteries were used to detect possible uneven distribution of the injected microspheres. The difference in count rate per ml blood per min between the samples from the two arteries was less than 10 per cent of the greater count rate.

The blood sample showing the lower count rate/flow ratio was chosen as reference. This was reasonable as in the tissues some 5—10 per cent of the microspheres of this particular size would pass through during one circulation (Ring *et al.* 1961). Thus the radioactivity in the blood samples in which all the microspheres were collected would be somewhat higher than in the tissue where only 90—95 per cent of the inflowing microspheres were settled.

The following formula was used for calculation of the unknown tissue blood flow (Q_T) in g/min per g wet tissue

$$Q_T = Q_A \cdot \frac{c.p.m._T}{c.p.m._A}$$

Q_A = blood flow in reference artery in g/min
c.p.m.$_T$ = counts per min per g tissue sample
c.p.m.$_A$ = counts per min in blood sampled from reference artery

Histological technique. 20 μ thick sections from the various tissues were prepared and stained with hematoxylin (Harris) and eosin for microscopic examination.

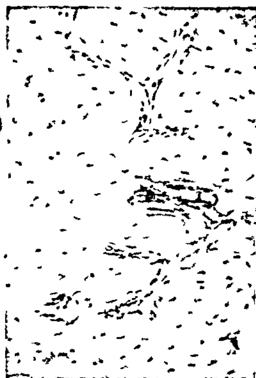
Results

The calculated data for blood flow through cortical bone of the femur diaphysis, the corresponding bone marrow and quadriceps tendon in 10 rabbits are presented in Table I.

Microscopic examination of the various tissues after injection of the microspheres revealed that few microspheres were lodged in cortical bone as compared to the other tissues. Fig. 3 shows a typical picture of one microsphere which is lodged in a vessel in a Haversian canal in the femur diaphysis. Very few aggregates were seen and never more than three microspheres in one aggregate.

TABLE I Regional blood flow values in 10 anesthetized adult rabbits

Expt no	Blood flow (g/min × g tissue)		
	Quadriceps fem tendon	Bone marrow fem diaphysis	Cortical bone fem diaphysis
1	0 094	0 194	0 013
2		0 229	0 016
3	0 112	0 148	0 001
4	0 113	0 621	0 012
5	0 115	0 265	0 008
6	0 010	0 258	0 004
7	0 017	0 120	0 001
8	0 014	0 112	0 005
9	0 076	0 253	0 013
10	0 057	0 301	0 022
Mean ± S D	0 068 ± 0 044	0 250 ± 0 146	0 010 ± 0 007



A



B

Fig 3 A and B *Longitudinal section from cortical bone of rabbit femoral diaphysis* A microsphere with a diameter of about 15 μ is lodged in a Haversian vessel At A 260× B 900×

Discussion

The method used seems suitable for quantitative determination of blood flow in inaccessible and minute tissue regions The introduction of the small number of microspheres needed to obtain a sufficient radioemission from tissue samples would hardly affect the over all circulatory state in our hind limb preparation The findings of Edlich *et al* (1968) that various microspheres with different physical properties show similar distribution within the gastric microcirculation support this view

The handling of such microspheres as well as the injection procedure is critical however *to avoid aggregation of the microspheres and to obtain an even distribution* in various tissue regions

The method is based on the assumption that a linear relationship exists between blood flow and radioactivity The blood flow through the abdominal aorta (Q) is equal to the sum of the blood flows in the various peripheral arteries which are coupled in series with this main stem artery Thus

$$Q = Q_1 + Q_2 + Q_3 + \dots + Q_n$$

Provided the spheres are evenly mixed with the blood in the aorta the concentration of the spheres (c) in a transverse section of the blood column is the same at any level Therefore the following equation is valid

$$Q \cdot c = Q_1 \cdot c + Q_2 \cdot c + Q_3 \cdot c + \dots + Q_n \cdot c$$

The amount of microspheres (or radioactivity) in the various parts of the arterial tree will therefore be directly proportional to the blood flow

A great practical advantage of the present method is the correlation to blood flow values obtained by direct sampling of blood from cannulas inserted into small arteries By this procedure the blood flow from one or more of the many parallel arterial ramifications (Q_x) is determined directly in ml per min By measuring the radioactivity in the same sample corresponding to this flow the product $Q_x \cdot c$ is determined

This procedure makes it unnecessary to determine the exact amount of injected microspheres Furthermore by simultaneous sampling of blood from two arteries it could be seen whether or not the microspheres were evenly distributed

Our results (Table I) show considerable variations in flow values from one animal to another within the same type of tissue As stated above this uneven distribution of the microspheres could hardly be due to an inadequate injection technique Other investigators using a similar technique (Neutze *et al* 1968) have also obtained considerable variations in resting flow values in various other tissues Thus it seems more likely that the variations show real differences between anesthetized but otherwise intact animals The size of the blood flow through rabbit femoral bone marrow detected in the present series of experiments is however of the same magnitude as that obtained by Michelsen (1968) using a perfusion technique

Very few data have been found as regards previous studies on blood flow thro tendons and cortical bone Brookes (1967) found a significantly higher rate

cortical blood flow in rat femur The discrepancy between his and our data may have several explanations Brookes' technique may have involved other structures like periosteal and endosteal tissue in addition to cortical bone Our findings indicate that blood flow through cortical bone in mature rabbits is extremely small This could have been due to anatomical peculiarities in cortical bone As already mentioned (see Methods), Brånemark found that the vessel diameter in the Haversian channels in rabbit tibia seldom exceeds 15 μ This might result in a relatively restricted entrance into the cortical bone for the microspheres (15 ± 5 μ in diameter) used in our series of experiments However, as judged from the microscopic examinations the relative amount of microspheres of extreme size (20 and 10 μ in diameter) trapped in cortical bone did not deviate significantly from that in femoral muscle A relative accumulation of microspheres in periosteal vessels was, however, regularly seen but this may merely indicate that these tissues are highly vascularized, rather than that the entrance to cortical vessels has been blocked The interpretation difficulties encountered here might to some extent be overcome by comparing the relative distribution in cortical bone and other tissues of microspheres of a smaller size for example with a diameter of 10 μ Sufficiently uniform microspheres of this dimension were not available however

The present technique could be applied experimentally to various problems concerning the circulatory physiology and pharmacology of bone and tendoligamentous tissue Furthermore the blood supply to various regions of fractured bones during the healing procedure could be extensively studied The circulatory state in other pathological conditions in bone and tendoligamentous tissue might also be evaluated

The financial support to this study by Norsk Medisinaldepots Fond is gratefully acknowledged We also want to thank Dr J G Iversen and Dr H Benestad for the preparation of the photomicrographs

References

BROOKES M and R G HARRISON The vascularization of the rabbit femur and tibiofibula *J Anat* 1957 *91* 61 72

BROOKES M Blood flow rates in compact and cancellous bone and bone marrow *J Anat* 1967 *101* 533 541

BLANCHARD R J W I GROTENHUIS J W LAFAVE and J F PERRY JR Blood supply to hepatic V2 carcinoma implants as measured by radioactivity microspheres *Proc Soc exp Biol N Y* 1965 *118* 465—468

BRÅNEMARK P I Vital microscopy of bone marrow in rabbit *Scand J clin Lab Invest* 1959 *11* Suppl 38 5 82

EDLICH R F I GROTENHUIS and R J BUCHIN Radioactive microspheres Effect of their physical properties on vascular distribution *Proc Soc exp Biol (N Y)* 1968 *128* 909—913

MICHELSEN K Hemodynamics of the bone marrow circulation *Acta physiol scand* 1968 *73* 264—280

NEUTZE J M F WYLER and A M RUDOLPH Use of radioactive microspheres to assess distribution of cardiac output in rabbits *Amer J Physiol* 1968 *215* 486—495

RING G C A S BLUM T KURBATOV W G MOSS and W SMITH Size of microspheres passing through pulmonary circuit in the dog *Amer J Physiol* 1961 *200* 1191 1196

RUDOLPH A M and M A HEYMANN The circulation of the fetus in utero Methods for studying distribution of blood flow cardiac output and organ blood flow *Circulat Res* 1967 *21* 163—184

YA P M, T GUZMAN M K LOKEN and J F PERRY Isotope localization with tagged microspheres *Surgery* 1961 *49* 644 650

Acta physiol scand 1970. 80. 45—49
From the Institute of Zoophysiology, Uppsala Sweden

The Incorporation of Cholesterol into the Nervous System of the Newly Hatched Chick

By

OLOF SVANBERG

Received 22 January 1970

Abstract

SVANBERG, O *The incorporation of cholesterol into the nervous system of the newly hatched chick* Acta physiol scand 1970 *80* 45—49

The deposition of cholesterol in the myelin of the central nervous system has been compared with that in *the myelin of the peripheral nervous system* C^{14}*-4-cholesterol was injected into* the yolk sacs of newly hatched chicks and whole body autoradiography was performed accord

Myelin contains large amounts of cholesterol Observations on the appearance of this easily analyzed substance may provide valuable information about the myelination process in the nervous system

The aim of the present investigation was to compare the incorporation of already synthesized cholesterol into the myelin in the central nervous system with that into the myelin in the peripheral nerves

Material and methods

A C^{14}-4-cholesterol suspension (Bergstrom and Wintersteiner 1941) was injected into the yolk sacs of newly hatched White Leghorn chicks C^{14} labelled cholesterol with a specific activity of 55 8 mci/mmole was obtained from the Radiochemical Centre Amersham England Each animal was given 3 µci After various periods of time up to nine days individual chicks were

before the films were developed

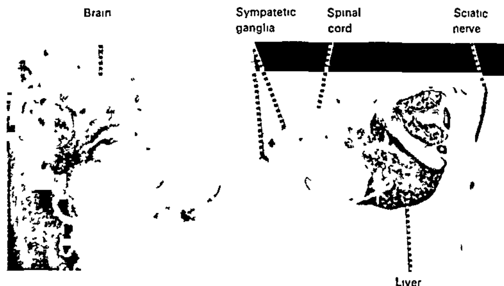


Fig 1 Autoradiogram from a 7 day-old chick injected with C^{14}-4 cholesterol at hatching Note the uptake (light areas) in the sciatic nerve and sympathetic ganglia

Pineal gland

Fig 2 Autoradiogram from the head of a 5 day-old chick injected with C^{14} 4 cholesterol at hatching Note the uptake (light area) in the pineal gland

Results

As will be seen from Fig 1 and 2 no radioactivity can be detected at any time in the brain and the spinal cord except in the pineal gland The peripheral nerves however, produce a heavy blackening on the films even after 2 to 3 days In the 3 day-old chick the sciatic nerve has incorporated more cholesterol than the muscles, for example Only the liver and the adrenal gland show higher activity After 7 days (Fig 1) radioactive cholesterol has accumulated still more in the sciatic nerve Also other parts of the peripheral nervous system for example the sympathetic ganglia and the brachial plexus appear heavily marked The concentration in the liver has decreased markedly which shows that the amount of cholesterol which was transported from the residual yolk sac during the period was small in relation to that transported from the liver After 8 and 9 days the changes noted are still more accentuated

Discussion

The lamellae of the myelin sheath are derived from successive layers of the plasma membranes of the glial cells (*cf* Cragg 1968) The myelination process is thus a membrane synthesis the precursors of which may either be transported to the nervous tissue or synthesized *in situ*

Central nervous system

Many workers think that there is a blood brain barrier which selectively excludes substances such as cholesterol from the brain Hence these substances have to be synthesized by the brain This opinion was put forward by Waelsch *et al* (1940) who also found that cholesterol synthesis only occurs in the brains of newborn rats Bloch *et al* (1943) could not find any trace of i v injected deuterium labelled cholesterol in the brains of adult dogs However other workers (Chevallier and Petit 1966 Appelgren 1967), using autoradiographic technique have shown that C^{14} cholesterol injected into adult rats is laid down in the brain but at a very slow rate Davison *et al* (1958) and Kritchevsky and Defendi (1962) extracted radioactive cholesterol from the brains of chicks after it had been injected into the residual yolk sac at hatching and they deny the existence of a blood brain barrier

The ability of the brains of newborn rats to synthesize cholesterol *in vitro* has been demonstrated by Srere *et al* (1950) McMillan *et al* (1957) also observed synthesis in the brains of adult rats but at a very much slower rate

It has been shown by the use of different techniques that myelination of the central nervous system of the chick starts during the second week of incubation and is finished 2 to 3 months after hatching (Mandel *et al* 1949 Bensted *et al* 1957 Davision *et al* 1958 Grafnetter *et al* 1965 Medda and Bose 1966) During this period the cholesterol content of the nervous tissue increases manyfold

The absence of C^{14}-4 cholesterol in the present material thus indicates that the bulk of the cholesterol that makes up the myelin sheaths is not derived from the

blood but is synthesized in the brain, probably by the oligodendroglial cells The reason why cholesterol, which appears in very high concentrations in the blood of the chick during the first week after hatching (Entenman *et al* 1940), is not utilized in the formation of the myelin sheaths but has to be synthesized *in situ* is not clear It may depend on a restricted ability of the cholesterol molecule to reach the oligodendroglial cells due to a 'blood brain barrier' in the wide sense as discussed by Dobbing (1968)

The discrepancy between the present results and those mentioned earlier may be explained by the presence of two pools of cholesterol, a very small one, not detected in this autoradiographic study which is transported to the brain, and a larger one which is synthesized by the glial cells

This idea is supported by the findings of Dobbings (1963), that after injection of C^{14} 4-cholesterol into young rats there is a close connection between the increment in the total cholesterol of the brain and that of the incorporation of radioactivity

The fact that the pineal gland shows a rather rapid uptake of cholesterol is surprising and requires further investigation Wislocki and Leduc (1952) showed that also other substances which are hindered by a 'blood brain barrier" penetrates into the pineal gland

Peripheral nervous system

Little is known about the deposition of cholesterol in peripheral nerves The first medullated fibres in the sciatic nerve of chicks are seen during the fifteenth day of incubation (Romanoff 1960) Foppen and Liuzzi (1968) showed that the amount of cholesterol in the sensory ganglia increases sharply during the second week of incubation

The results obtained by the present author indicate that myelination continues in the peripheral nerves during the first week after hatching and imply the incorporation of already synthesized cholesterol It is accordingly possible for cholesterol to pass freely between the blood and the glial cells of the peripheral nerves

These findings — that the central and the peripheral nervous systems differ so distinctly as regards the deposition of presynthesized cholesterol in the myelin — have not been reported earlier In this connection it is of interest that in the central nervous system oligodendroglial cells form the myelin sheath while in the peripheral nerves the cells of Schwann do this

The author wishes to express his thanks to Professor P E Lindahl for helpful discussions and valuable criticism

This work has been supported by grants from the Hierta Retzius Foundation and from the Faculty of Science of the University of Uppsala Sweden

References

APPELGREN, L-E Sites of steroid hormone formation *Acta physiol scand* 1967 *71*, Suppl 301 1—108

P ... WRIGHT, Neuro-
J Embryol exp

I ... aqueous solution

BLOCK, K ... and D RITTENBERG, The biological conversion of cholesterol to cholic acid *J* ... *Chem* 1943 *149* 511—517

CHEVALIER, F and ... PETIT, Incorporation of cholesterol into the central nervous system and its autoradiographic localization *Exp Neurol* 1966 *16* 250—254

CRAIG ...

DAVIS ...
disposal of /4-^{14}C/cholesterol in the brain of growing chicks *J Neurochem* 1958 *3* 89—94

DOBBING, J, The entry of cholesterol into rat brain during development *J Neurochem* 1963 *10* 739—742

DOBBING, J, *The blood-brain barrier* In "Applied Neurochemistry", ed A N Davison and ...

E ...

Fc ...

C ... the chicken brain
...—149

K ... olesterol, sitosterol, lanosterol) in the brain and other organs of the growing chicken *J Neurochem* 1962 *9* 421—425

MANDEL, P, R BIETH and R STOLL, La repartition des diverses fractions lipidiques dans le cerveau de l'embryon de poulet durant la seconde partie de l'incubation *C R Soc Biol* (Paris) 1949 *143* 1224—1226

McMILLAN, P J, G W DOUGLAS and R A MORTENSEN, Incorporation of C^{14} of acetate-1 C^{14} and pyruvate 2 C^{14} into brain cholesterol in the intact rat *Proc Soc exp Biol* (N Y) 1957 *96* 738—740

...

WISLOCKI G B and ELIZABETH H LEDUC, Vital staining of the hematoencephalic barrier by silver nitrate and trypan blue, and cytological comparisons of the neurohypophysis, pineal body area postrema, intercolumner tubercule and supraoptic crest *J comp Neurol* 1952 *96* 371—413

Acta physiol. scand. 1970, 80. 50—60

From the Department of Physiology, University of Goteborg, Sweden

Plasma Skimming in the Intestinal Tract

By

MATS JODAL AND OVE LUNDGREN

Received 26 January 1970

Abstract

JODAL, M and O LUNDGREN *Plasma skimming in the intestinal tract* Acta physiol scand 1970 *80* 50—60

The "tissue hematocrit" of the different wall layers of the small and large bowel of the cat was studied in three [illegible] during "resting" blood flow of the denervated intestine, [illegible] propylnoradrenaline and during nervous vasoconstriction [illegible] ated from the distribution of ^{51}Cr labelled red cells and [illegible] labelled human serum albumin It was demonstrated that the "tissue hematocrit" of the mucosa of the small intestine only amounted to 50—60 per cent of the arterial hematocrit while the "muscularis hematocrit" was 90 per cent of the arterial Similar results were obtained in the three different experimental situations The low mucosal "tissue hematocrit could neither be explained by an extravascular accumulation of ^{125}I labelled albumin nor by different degrees of axial streaming of erythrocytes in the different wall layers The observations are therefore taken to indicate the presence of a plasma skimming in the small intestine of the cat In the colon the "tissue hematocrit" of the mucosa was only slightly lower than that of the submucosa, the muscularis or the arterial blood indicating that no or only a slight plasma skimming occurs in this organ

It is well established that blood cells pass through the vasculature of a tissue faster than plasma because of the tendency of the cells to flow in the fast axial stream in the vessels with diameters below 200—250 μm Hence, there exists a relatively cell free layer of blood close to the vessel wall at least when flow rate is reasonably high The plasma may in certain situations be "skimmed" off at branching points resulting in a difference in hematocrit in the two dividing vessels Two factors have been proposed to determine the extent of plasma skimming, *i e* the ratio of the linear rates of blood flow in the two dividing vessels and the angle between the branching vessels (Krogh 1922 Pappenheimer and Kinter 1956 Palmer 1959, 1965)

A plasma skimming (or cell separation) no doubt occurs at certain points in all vascular beds but it will only become haemodynamically significant if a tissue is predominantly supplied by blood containing an abnormal proportion of blood cells Thus, Pappenheimer and Kinter (1956) proposed such a plasma skimming mechanism in the kidney cortex as an explanation for the autoregulation of blood flow and glomerular filtration rate The cell separation was supposed to take place in the in-

terlobular and peritubular vessels However, this ingenious hypothesis has now been abandoned, at least as it was originally proposed by Pappenheimer and Kinter (Ulfendahl 1962, Selkurt 1963) The blood flowing through the vasa recta loops in the papilla seems, however, to have a comparatively low hematocrit (Ulfendahl 1962)

The vascular architecture of the gastrointestinal tract indicates the possible existence of a plasma skimming mechanism since the vascular supply to the mucosa seems to branch off from the dense submucosal vascular network at a right angle (Hou-Jensen 1931). Furthermore, the submucosal vessels have a diameter of approximately 50—100 μm (Mall 1888, Hou-Jensen 1931, Baez 1959) a size at which there seems to exist a large cell poor plasma zone close to the vascular wall (Fåhraeus 1929) The present study was undertaken to test this hypothesis in the small intestine and in the colon Some preliminary results have been published previously (Jodal and Lundgren 1968)

Methods

A Operative procedures and determination of blood flow

The experiments were performed on 34 cats anesthetized with chloralose (40—60 mg/kg b w) after induction with ether The animals had been deprived of food for at least 24 hrs and had no obvious signs of infection in the gastrointestinal tract

After insertion of a tracheal cannula, the abdomen was opened in the midline and the greater omentum and the spleen were extirpated In experiments performed on the *small intestine* 2—3 segments of the gut were isolated and the remainder of the intestinal tract was extirpated The lumen of the segments were flushed with saline at 38° C The intestinal segments were then cut open by thermocautery along the antimesenteric border

After heparinizing the animal (3—5 mg/kg b w) the right femoral artery was cannulated and connected to a mercury manometer to record mean arterial blood pressure The superior mesenteric vein, draining all the blood from the isolated intestinal segments and its remaining lymph nodes, was cannulated and connected to an optical drop recorder unit operating an ordinate writer recording on smoked kymograph paper The venous blood was returned to the animal *via* a funnel connected to a catheter in the right jugular vein

In experiments performed on the *colon* the operative procedure was in principle similar to that described above (*cf* Hultén, Jodal and Lundgren 1969 a), although the large bowel was only divided into two segments The colon was cut at its entrance into the pelvis Hence, the [illegible]

used since it relaxes the intestinal smooth muscle as well as the vascular smooth muscle (*cf* Folkow, Lundgren and Wallentin 1963) The drug was given at a rate of 5—10 μg/min

B Nervous stimulations

The sympathetic vasoconstrictor fibre supply to the intestinal tract was stimulated in the following way The *splanchnic nerves*, innervating the small intestine and the proximal part of the colon (Hultén Jodal and Lundgren 1969 a), were cut on both sides beneath the diaphragm and the peripheral ends were mounted on silver ring electrodes Supramaximal square wave pulses (5—8 V, 5 msec) were delivered from a Grass stimulator, model S4 E The stimulus frequency was set at 4 or 8 imp/sec

The *lumbar colonic nerves* innervating the distal part of the colon (Hulten, Jodal and Lundgren 1969 a), were dissected free from the inferior mesenteric artery, cut and their distal ends were placed on silver ring electrodes Since these nerve fibres are postganglionic, a stimulation strength of 11—13 V was used, frequencies being the same as above

To eliminate the influence of the cathecolamines released from the adrenals, these organs were either denervated or excluded from the circulation by ligatures Atropine was given in most experiments (1 mg/kg b w)

C Isotope techniques

^{51}Cr labelled red cells and ^{125}I labelled human serum albumin was utilized to determine tissue hematocrit in the different wall layers of the intestinal tract To label the red cells with ^{51}Cr 8—10 ml of blood was drawn from the animal The erytrocytes were washed twice with saline and incubated with 400—800 μC $Na^{51}CrO_4$ (purchased from AB Atomenergi Studsvik Sweden) for 2 hrs at about 30° C (for technical details see Owen 1959) After three washings of the blood cells with saline ^{125}I labelled human serum albumin (200—400 μC purchased from AB Atomenergi Studsvik Sweden) and the serum solution originally obtained from the cats blood was added to the labelled red cells The labelled albumin solution was usually used within 10 days after delivery and in no experiment was the solution older than 1 month

The labelled blood solution was injected i v Within 1—7 min after the administration the tissue segments were rapidly extirpated and immediately frozen in an acetone dry ice mixture The arteries and veins supplying the tissue segments were simultaneously ligated just prior to the extirpation An arterial blood sample was drawn when the segments were taken *via* a catheter in the aortic arch

Two flat parts from each frozen tissue segment were selected and cut in a cryostat into a number of transverse sections 100—400 μm thick from the mucosal surface towards the serosa When sectioning the tissue segments it was easy to distinguish the mucosa and the muscularis The submucosal tissue was however, seldom obtained entirely free of mucosa and/or muscularis Since the submucosa constitutes only a small fraction of the intestinal wall (about 10—20 per cent) a comparatively large error was thus introduced. To indicate this the word submucosa has been put in quotation marks

Each tissue section was put into a test tube together with 2 ml concentrated sulphuric acid and left for digestion of the tissue for at least 12 hrs The relative amounts of ^{125}I and ^{51}Cr reflecting the amount of plasma and red cells respectively were determined by means of a well type scintillation detector coupled to a spectrometer (Packard Auto-Gamma Spectrometer series 410 A) and a scaler Pulses were counted corresponding to γ-energies at the photopeaks of the two isotopes used in the present study (^{125}I 28 keV, ^{51}Cr 325 keV) The counting rates in the "^{125}I-channel" were corrected for the contribution of radioactivity emanating from ^{51}Cr (*cf* Quimby and Feitelberg 1963 p 269) The time of counting was prolonged so that the coefficient of variation amounted to 3—5 per cent as determined by the equation given by Quimby and Feitelberg (1963 p 207)

In each experiment the relative amounts of ^{51}Cr and ^{125}I was determined in the arterial blood sample drawn at the time of extirpation of the tissue segments (see above) This was treated in a similar way as described above Blood hematocrit was also determined in all experiments except in a few early ones in which the mean value of all determinations made in this series of experiments (35 %) was used Heparinized capillary tubes were filled with blood in one end sealed by flame and centrifugated at 5000 rpm for 5 min No correction for trapped plasma was made

D Calculations

From the arterial hematocrit and from the ratio between $^{51}Cr/^{125}I$ in tissue $(Cr/I)_t$ and $^{51}Cr/^{125}I$ in blood $(Cr/I)_b$ it was possible to calculate "tissue hematocrit" utilizing the diagram of Fig 1 For example if the above mentioned ratio was close to 0 8 and arterial hematocrit was 35 per cent "tissue hematocrit" could be estimated from Fig 1 (dotted line) to be 30 per cent. The arterial "isohematocrits" of Fig 1 were derived from the ratio $(Cr/I)_t$ $(Cr/I)_b$ using known values for tissue and arterial hematocrits

Results

A Small intestine

The "tissue hematocrit" of the different wall layers of the intestinal tract was studied during three different experimental conditions *i e* during resting blood flow of the denervated intestine during vasodilatation induced by isopropylnoradrenaline and during nervous vasoconstriction The infusion of isopropylnoradrenaline increased total venous outflow 3—5 times The vasoconstriction experiments were performed during the "steady state" phase of a nervous vasoconstriction (*cf* Folkow *et al* 1964, Hulten Jodal and Lundgren 1969 b) The results of experiments per

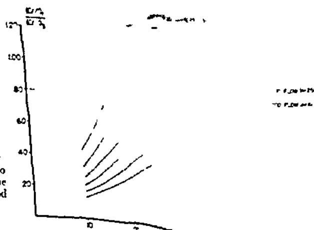


Fig 1 Diagram utilized to calculate "tissue hematocrit" from arterial hematocrit and the ratio between $^{51}Cr/^{125}I$ in the tissue $(Cr/I)_t$ and $^{51}Cr/^{125}I$ in blood $(Cr/I)_b$ For details, see text

ie small
tributed

formed on the small intestine are illustrated in Fig 2. T
"tissue hematocrit" of each wall layer was calculated
tissue sections With regard to the mucosa, the tiss
2/3—3/4 of this wall layer were chosen for the dete
admixture from blood in the large submucosal vessels l in the
of the mucosa represents probably predominantly the results
this layer xtensive

It is evident from Fig 2 that the "tissue hemato erficial
lower ($p<0.001$) than that of the "submucosa", t
blood at the different levels of vascular tone studied er than
about 35 per cent, the "tissue hematocrit" of the evels of
20 per cent Thus, these observations suggest the means

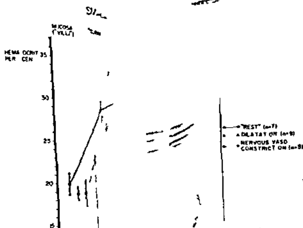


Fig 2 "Tissue hematocrit" of the different wall layers of the small intestine during "resting" conditions during vasodilatation induced by intravascular infusion of isopropylnoradrenaline and during vasoconstriction produced by activation of the regional sympathetic vasoconstrictor fibres The arterial hematocrit of the corresponding experiments are also shown in the figure Vertical lines indicate ± S E M

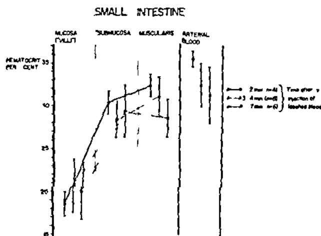


Fig 3 'Tissue hematocrit of the different wall layers of intestinal segments extirpated at different times after the injection of the labelled blood The experiments *were performed during "resting* conditions (*cf* Fig 2) The arterial hematocrit of the corresponding experiments are also shown Vertical lines indicate ± S E M

skimming mechanism in the small intestine The 'tissue hematocrit" of the muscularis on the other hand, was estimated to be approximately 90 per cent of the arterial hematocrit

The results of Fig 2 indicate, furthermore that the cell separation may be more efficient during nervous vasoconstriction since the 'tissue hematocrit" of the 'submucosa is higher during sympathetic nervous influence than during 'resting conditions or vasodilatation It should be pointed out, however, that the error in calculating the tissue hematocrit" of the submucosa' is comparatively large (see *Methods section C*)

The results obtained during 'resting conditions were divided into three groups according to the time between extirpation and intravenous injection of the labelled blood This was made in 18 tissue sections where the exact time of extirpation was known The results are illustrated in Fig 3 It is evident that there exists no significant difference between the results obtained at the different times of extirpation

The tendency of blood cells to move in the fast axial stream of a blood vessel is less pronounced at lower linear rates of blood flow Consequently, it should be possible to reduce the extent of a plasma skimming by lowering the rate of blood flow This was attempted in two experiments by means of a screw clamp around the aorta just proximal to the coeliac artery By lowering the arterial blood pressure to about 30 mm Hg thereby reducing the blood flow to 1/3—1/2 of control, the tissue hematocrit of the mucosa rose to the same order of magnitude as in the other two wall layers These results are illustrated in Fig 4 in which the "resting' curve of Fig 2 is also included as a comparison

In two control experiments the labelled blood was infused close intra arterially into the superior mesenteric artery and the total venous outflow from the gut was collected continuously to prevent recirculation of the tracers 1/2—1 min after the end of the infusion an intestinal segment was extirpated and the content of ^{51}Cr and

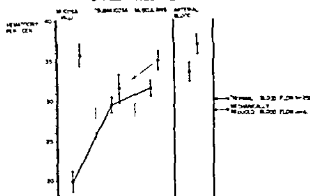


Fig 4 'Tissue hematocrit' of the different wall layers of the small intestine during "normal" blood flow perfusion and during reduced intestinal blood flow induced by partial occlusion of the aorta. The arterial hematocrit of the corresponding experiments are also shown Vertical lines indicate ± S E M

¹³¹I in the different wall layers was determined in the usual manner The small amount of radioactivity remaining in the intestinal tissue was evenly distributed throughout the different layers of the small intestine

B Colon

The "tissue hematocrit' of the different wall layers of the colon was studied in the same experimental situations as described above for the small intestine The results are schematically illustrated in Fig 5 To avoid admixture of blood in the extensive submucosal network with blood from the thin colonic mucosa, only the superficial tissue sections were included in the calculation of mucosal 'tissue hematocrit'

Fig 5 indicates that the 'tissue hematocrit' in the mucosa is slightly lower than that of the other two tissue layers and the arterial blood at all the different levels of blood flow The mucosal "tissue hematocrit" of the colon is, however, by no means as low as that in the mucosa of the small intestine (*cf* Fig 2 and 5)

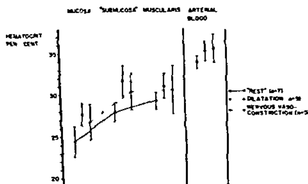


Fig 5 Tissue hematocrit of the different wall layers of the colon during resting" conditions during vasodilatation induced by intravascular infusion of isopropyl noradrenaline and during vasoconstriction produced by activation of the regional sympathetic vasoconstrictor fibres The arterial hematocrit of the corresponding experiments are also shown Vertical lines indicate ± S E M

Discussion

The results of the present study strongly suggest that the mucosa of the small intestine is perfused by blood of low hematocrit reflected in a low $^{51}Cr/^{125}I$ ratio in the superficial 2/3—3/4 of the mucosa as compared to that of blood (Fig. 2). This finding observed at different levels of vascular tone cannot be explained by any accumulation of free ^{125}I iodide in the tissue and/or leakage of albumin—^{125}I into the interstitial space since control experiments clearly indicated an even distribution of radioactive iodide throughout the wall of the small intestine. In these control experiments the tracer was administered close intraarterially to ensure a maximal delivery of the radioactive solution to the vascular bed of the small intestine and the intestinal segments were extirpated 1/2—1 min after vascular perfusion with unlabelled blood. Furthermore, no significant difference was found in tissue hematocrit between intestinal segments extirpated at different times after the administration of the labelled blood (Fig. 3).

It was also clearly demonstrated in the present study that increasing total intestinal blood flow 3—5 times and thus increasing linear rate of flow in the mucosa by at least twofold (Biber, Lundgren and Svanvik 1969) did not alter significantly the tissue hematocrit of the mucosa. This observation seems to rule out the possibility that the low tissue hematocrit of the mucosa was largely due to regional differences in the extent of axial streaming of erythrocytes. This conclusion may also be further strengthened by the failure to demonstrate any low tissue hematocrit in the colonic mucosa (Fig. 5).

The vascular architecture of the small intestine would facilitate, as mentioned in the introduction, the occurrence of plasma skimming (cell separation) in the extensive vascular networks of the small intestine located mainly in the submucosa. It is therefore proposed that such a mechanism explains the observations reported above. This conclusion is furthermore strengthened by the observation that it was possible to abolish the uneven distribution of tissue hematocrit in the small intestine by mechanically reducing total intestinal blood flow (Fig. 4). This procedure greatly lowered the linear rate of blood flow and hence the extent of axial flow of blood cells in the vessels.

The results obtained on the colon (Fig. 5) indicate that there occurs no or only a slight plasma skimming in the submucosa of this organ. The reason for this difference between the colon and the small intestine is not known. It should be pointed out, however, that the extent of the possible plasma skimming occurring in the colon may be underestimated with the present technique since the colonic mucosa is thin (1/2—1/3 of the thickness of the small intestinal mucosa) and thus more difficult to obtain free of submucosa at the histological sectioning.

Fig. 6 schematically suggests one possible anatomical arrangement which might cause the plasma skimming observed in the small intestine in the present study. According to this hypothesis the plasma skimming occurs in one stage, e.g. exclusively in the submucosal network. The blood of low hematocrit would be diverted to a villus

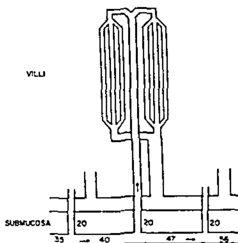


Fig 6

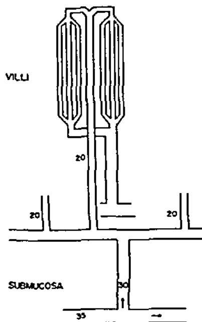


Fig 7

Fig 6 Proposed anatomical arrangement to explain the plasma skimming occurring in the small intestine The "skimming" is assumed to take place in one "stage" Numbers indicate hematocrit For details, see text

Fig 7 Proposed anatomical arrangement to explain the plasma skimming occurring in the small intestine The "skimming" is assumed to take place in two "stages" Numbers indicate hematocrit For details see text

artery To what extent plasma skimming also occurs into vessels supplying the crypts is not known and cannot be determined with the present technique

In Fig 6 an attempt has also been made to estimate the increase of hematocrit that may occur in the submucosal vessel as low hematocrit blood is "skimmed off" It is assumed that the hematocrit in the afferent submucosal artery is 35 per cent and that blood flow in the vessels supplying the mucosa is 1/4 of that in the submucosal vessel It can then be calculated that the hematocrit of arterial blood, after passing three branching points for mucosal vessels will amount to approximately 56 per cent This hematocrit is the maximum one that theoretically can exist in a blood vessel without deforming red cells (Burton 1965)

Fig 7 illustrates another possible anatomical arrangement that might explain the cell separation in the small intestine According to this hypothesis plasma skimming occurs in "stages", *e g* first in the submucosa and then in the vascular network surrounding the crypts This hypothesis is in accordance with the vascular anatomy of the small intestine as described by Heller (1872) Baez (1959) and Reynolds Brim and Sheehy (1967)

The above mentioned considerations pose the question Which vessels receive the blood of a high hematocrit? No attempts have been made in the present study to localize these vessels and the following discussion is speculative Three possible routes seem to exist (1) Mall (1888) and Spanner (1932) described a peculiar vascular structure in the intestinal submucosa named Venenballchen They consist of arterioles containing longitudinal smooth muscles which arborize into a dense meshwork of thin walled veins The functional significance of these structures is unknown (2) The bases of the crypts where cell renewal takes place is evidently a part of the intestinal wall of high metabolic activity This is reflected by the fact that the bases of the crypts are extremely well vascularized (Lundgren 1967) It is possible that the oxygen delivery to this region is further improved by a perfusion of a high hematocrit blood (3) The high hematocrit blood may possibly be diverted through *the arterio venous shunts described by Baez (1959) at the bases of the villi*

This study was prompted by anatomical considerations concerning the vasculature of the intestinal tract and the cell separation hypothesis described above is related to anatomical pecularities of the vasculature Another concept has been introduced by Palmer 1959 1965) who favours the view that plasma skimming occurs when the ratio of the linear rates of blood flow in two dividing vessels reaches a certain value even in the absence of a right angle branching point Since there are no detailed data available concerning linear rates of blood flow in the intramural intestinal vessels it seems impossible to evaluate the hypothesis of Palmer in the light of the results of the present study

The small intestinal vascular bed is unique among the major beds of the organism in the sense that an extensive capillary network is situated directly below a thin monolayered epithelium Furthermore the contents of the intestinal lumen is variable and may present the blood in the intestinal capillaries to sudden and large variation in osmolarity and concentration of various solutes The osmolarity in the intestinal lumen is of particular importance for the blood cells since hypotonicity may cause lysis of cells It has also been demonstrated that after drinking of large volumes of water as much as 3 g free hemoglobin can be found per 100 ml portal venous plasma (Davenport 1966) The cell separation demonstrated in this study with a cell poor blood reaching the mucosa no doubt tends to reduce the risk of such hemolysis

It has been proposed that there exists a decreasing gradient of oxygen pressure from the bases towards the tips of the intestinal villi due to a countercurrent exchange of oxygen in the intestinal mucosa (Kampp Lundgren and Nilsson 1968 The plasma skimming described in this study will further diminish the oxygen delivery to the cells in the apical parts of the intestinal villi This might be of particular importance in situations of long periods of nervous vasoconstriction *e g* during shock Microdiver studies on cells from the bases and from the tips of rat villi indicate however that the oxygen consumption of the basal cells is higher than that of the apical ones (Hamberger and Lundgren to be published) possibly due to an adaptation to the oxygen tension prevailing at the tips of the villi

It was proposed in the series of papers by Pappenheimer and Kinter (1956) that

the cell separation presumed to occur in the kidney cortex, explained the autoregulation exhibited by kidney blood flow The intestinal vascular bed is also characterized by blood flow autoregulation The observation that the extent of cell separation was diminished by mechanically reducing total intestinal blood flow (i e decreasing blood pressure below the autoregulatory range) would seem to favour the interpretation that plasma skimming may also be one factor responsible for the autoregulation of intestinal blood flow However, the experimental work by Johnson (1964) strongly indicates that the intestinal autoregulation is mainly a myogenic phenomenon This conclusion is also supported by the observation that vasodilatation greatly reduces the autoregulation of blood flow (Johnson 1959) but has no effect on the extent of plasma skimming (Fig 2)

The excellent technical assistance of Mrs Dagny Johansson and Miss Lisbeth Larsson is gratefully acknowledged

This research has been sponsored in part by the AIR FORCE OFFICE OF SCIENTIFIC RESEARCH OAR through the European Office of Aerospace Research United States Air Force under Contract No F61052-68-C-0044, by a grant from US Public Health Service HE-05675-08), from the Swedish Medical Research Council (B70-14X 16-06B and B70-14X 2855 01 and from the Faculty of Medicine, University of Goteborg

References

BAEZ S *Microcirculation in the intramural vessels of the small intestine in the rat* In The Microcirculation The University of Illinois Press Urbana 1959

BIBER B O LUNDGREN and J SVANVIK An indicator-dilution technique for studying mean transit time and blood flow in the mucosa-submucosa of the small intestine *Acta physiol scand* 1969 Suppl 330 99

BURTON A C *Physiology and biophysics of the circulation* Year Book Medical Publishers Inc Chicago 1965

DAVENPORT H W *Physiology of the digestive tract* Ed 2 Year Book Medical Publishers Inc Chicago 1966

FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 57 270—283

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of grad[illegible] nce and capacitance vessels *Acta* [illegible]

FAHRAE[illegible] 1929 *9* 241—274

HELLE[illegible] Bios 1872 *24* 165—171

HOU J[illegible] ntestinum Ileum *Z Anat Entwickl Gesch* 1931 *94* 68—93

HULTEN L M JODAL and O LUNDGREN Extrinsic nervous control of colonic blood flow *Acta physiol scand* 1969 a Suppl 335 39—50

HULTEN L M JODAL and O LUNDGREN Local and nervous control of the consecutive vascular sections of the colon *Acta physiol scand* 1969 b Suppl 335 51—64

JODAL M and O LUNDGREN Plasma skimming in the intestinal wall In *International Conference on Microcirculation* 1968 Abstracts p 90

JOHNSON P C Myogenic nature of increase in intestinal vascular resistance with venous pressure elevation *Circulat Res* 1959 7 992—999

JOHNSON P C Origin localization and homeostatic significance of autoregulation in the intestine *Circulat Res* 1964 *14* Suppl 1 225—232

KAMPP M O LUNDGREN and N J NILSON Extravascular shunting of oxygen in the small intestine of the cat *Acta physiol scand* 1968 *72* 396—403

KROGH A *The anatomy and physiology of capillaries* Yale University Press New Haven 1922

LUNDGREN O Studies on blood flow distribution and countercurrent exchange in the small intestine *Acta physiol scand* 1967 Suppl 303

MALL J P, Die Blut- und Lymphwege in Dünndarm des Hundes *Abh sächs Ges Wiss* 1888 *14* 153—189

OWEN, C A *Diagnostic Radioisotopes* Charles C Thomas Publ, Springfield, Ill 1959

PALMER A A, A study of blood flow in minute vessels of the pancreatic region of the rat with reference to intermittent corpuscular flow in individual capillaries *Quart J exp Physiol* 1959 *44* 149—159

PALMER A A, Axial drift of cells and partial plasma skimming in blood flowing through glass slits *Amer J Physiol* 1965 *209* 1115—1122

PAPPENHEIMER, J R and W B KINTER, Hematocrit ratio of blood within the mammalian kidney and its significance for renal hemodynamics *Amer J Physiol* 1956 *185* 377—390

QUIMBY E H and S FEITELBERG, *Radioactive isotopes in medicine and biology* Lea and Febiger Philadelphia 1963

REYNOLDS D G, J BRIM and T W SHEEHY, The vascular architecture of the small intestinal mucosa of the monkey (*Macaca mulatta*) *Anat Rec* 1967 *159* 211—218

SELKURT E E The renal circulation *Handbook of Physiology* Sect 2, *Vol II* 1963 1457—1516

SPANNER R, Neue Befunde uber die Blutwege der Darmwand und ihre funktionelle Bedeutung *Morph Jb* 1932 *69* 394—454

ULFENDAHL, H R, Distribution of red cells and plasma in rabbit and cat kidneys *Acta physiol scand* 1962 *56* 42—60

Acta physiol. scand. 1970. 80. 61—72

From the Department of Physiology, University of Göteborg, Sweden

Blood Flow through Limb Muscles during Heavy Rhythmic Exercise

By

B Folkow, P. Gaskell[1] and B A Waaler[2]

Received 27 January 1970

Abstract

Folkow, B P. Gaskell and B A Waaler *Blood flow through limb muscles during heavy rhythmic exercise* Acta physiol scand 1970 *80* 61—72

msec) impulse train once per sec, a pattern mimicking that during running Rhythmic contractions of this type led to a characteristic blood flow pattern through the muscles Arterial inflow to the muscle vascular bed occurred only in between contractions, whereas nearly all the venous

importance also in the dependent limbs of man

The blood flow through rhythmically contracting muscles is mechanically interfered with in a complex way During each contraction phase vessels will be more or less compressed This vascular compression is known to cause a temporary reduction in inflow to the muscles, and a temporary reduction in their venous blood content (Barcroft and Dornhurst 1949) The mechanism of rhythmic muscular contractions, repeatedly emptying the veins towards the heart, is often referred to as "the muscle pump" It is generally accepted that this muscle pump effect is of great circulatory importance particularly in a dependent limb, since it promotes the venous return to the heart and prevents the pooling of large amounts of blood in the venous system Stegall (1966) has pointed to another important aspect of this pump mechanism

[1] Present address Department of Physiology, University of Manitoba, Winnipeg, Canada.
[2] Present address Department of Physiology, University of Oslo, Oslo, Norway

and has calculated that muscle pumping of blood by the lower limbs contributes more than 30 % of the energy required to circulate blood during running

It appeared to us that the muscle pump effect could have still one more important circulatory effect, namely that of promoting local muscle flow by intermittently lowering venous pressure and thereby increasing the effective perfusion pressure of the muscle vascular bed It is undoubtedly so that local transmural venous pressure becomes somewhat reduced subsequent to a contraction of a muscle group, with emptying of the muscle veins It is thus well known that rhythmic muscular contractions in a dependent limb result in a considerable lowering of transmural pressure in the subcutaneous veins (Walker and Longland 1950, Pollach and Wood 1949 Stegall 1966) and also in the deeper veins (Hojensgård and Sturup 1952, Ludbrook 1962 Stegall 1966) Reduction in transmural pressure in these veins must again reflect a reduction in transmural pressure in veins within the muscles themselves An important question thus appeared to be under which circumstances and to what extent such a mechanism may play a role Firstly increases in muscle perfusion pressure can be expected to influence blood flow markedly only in situations where the vessels are maximally dilated In all other circumstances local adjustments of the resistance vessels would be a dominating factor in determining actual flow Secondly an effect of this type would be potentially important only in situations where transmural venous pressure is high so that the possibility of a quantitatively marked reduction is present The conditions for the efficient operation of a mechanism of this type thus seems to be present in the dependent limbs of healthy individuals performing heavy rhythmic exercise Here all the reserves inherent in exercise vasodilatation are fully mobilized Also blood flow is temporarily interrupted or reduced during the muscle contractions thereby impeding the blood supply to the muscle tissue Any increase in effective perfusion pressure in periods between contractions would result in an important gain in blood supply to the muscles It is not known however how complete is the emptying of the veins during a rapid phasic muscular contraction Nor is it known how quickly in such a situation the huge blood inflow subsequent to an emptying muscle contraction will fill up the vessels of the venous bed raising the venous pressure again towards its original transmural value In other words it is not known for how long a lowered venous pressure may be maintained in the muscle during maximal blood flow At low flows on the other hand this mechanism operates efficiently (Allwood 1957)

The main intention with the present study has been to analyse in model experiments how much might be gained in effective perfusion pressure in muscles of a limb performing heavy rhythmic exercise Particular attention has been paid to the situation in dependent limbs where the most marked phasic lowering of venous pressure, and hence the greatest gain in perfusion pressure could be expected

The experiments were performed on cats using in most tests an isolated calf muscle preparation (Kjellmer 1964) It could then be shown that rhythmic muscular contractions may considerably decrease mean muscular venous pressures and proportionally increase flow in the situation where there is maximal exercise vasodilatation

This effect of the muscle pump was greatly increased when the limb was lowered relatively to the body, so that vascular transmural pressures increased A preliminary report of the results obtained has been given previously (Folkow, Gaskell and Waaler 1969)

Methods

[illegible]

50 mg/kg The anesthesia was maintained with small doses of chloralose or of pentobarbitone (Nembutal®, Abbott, 1—2 mg/kg)

1 In introductory experiments an attempt was made to follow directly the venous pressure in a small calf muscle vein during series of rhythmic contractions in that muscle, strong enough to produce maximal vasodilatation These initial experiments were carried out on limbs where as little dissection as possible had been done The right femoral artery and vein were exposed [illegible]

[illegible]

thus take place The whole limb was kept in the desired position by means of a string around the paw which was again fastened to a bar

The sciatic nerve was reached through an incision in the thigh The nerve was cut and its distal end placed in a circular, bipolar electrode By stimulation of the sciatic nerve the leg was induced to perform rhythmic contractions Regularly repeated trains of impulses were given usually with one impulse train being applied per sec Each single stimulus was given at 4 V for 0 2 msec The duration of the impulse trains was varied from 50 to 400 msec and the stimulus frequency during the impulse trains was varied between 10 and 100 per sec

Only [illegible] and d [illegible] cannul [illegible] openin [illegible] would most probably have disturbed the function of the muscle pump, the effect of which we wanted to study The one suitable venous system runs on the dorso-lateral surface of the calf muscles underneath the outer facia The main superficial vein which was very small near the ankle received several deep branches from muscles underneath, and at the middle of the calf it had usually reached such a size that it was possible to cannulate it with a polyethylene catheter the tip of which had an external diameter of 0 3—0 5 mm. A constant slow saline in fusion was kept going through the catheter which was connected to a pressure transducer

2 In a second set of very similar experiments the isolated calf muscle preparation proper was used with blood flow and pressure measurements being carried out as described above

3 In a third set of experiments a more quantitative evaluation of the effect of the muscle pump on the flow through the contracting muscles was attempted, particularly with the limb in a dependent position Isolated calf muscle preparations were employed, and the limb was completely severed from the body by cutting the femoral bone The bone marrow cavity was packed with cotton wool soaked in silicon grease, to prevent blood leakage A relatively wide plastic tubing connected the left femoral artery of the animal with that of the limb Part of the blood from the left femoral artery was led back to the jugular vein of the animal via a Starling resistance This arrangement made it possible to maintain a constant mean arterial pressure to the limb during tests A similar tubing led the blood from the femoral vein of the preparation back to the femoral vein on the left side Both the arterial inflow and the venous outflow pressures were measured close to the limb, using Statham P23 transducers. Phasic venous out-

flow from the preparation was measured with an electromagnetic flowmeter During some tests the flow probe was placed on the arterial side, enabling phasic arterial inflow to be followed during those tests

Flow and pressures were followed during and immediately after periods of rhythmic contractions in these preparations When maximal exercise vasodilatation had been achieved the position of the leg relative to the body was altered whilst the muscles were still rhythmicall contracting thus increasing or decreasing the transmural vascular pressures In most tests a lowering of the leg which increased transmural pressure by 25 mm Hg was employed

Nerve stimulations were carried out as described for the first set of experiments The severed
derately flexed a

of the record m
s were converted
to values of flow per unit time by weighing and comparing with cut out paper areas corresponding to conditions of known levels of steady flow

Results

The effectiveness of the muscle pump in promoting blood flow through working muscles will depend on the degree of lowering of venous pressure between contractions An initial problem then was how much and for how long venous pressure would be reduced in the intervals between rhythmic contractions at maximal flow levels In the introductory experiments carried out on the relatively intact leg the first aim was to analyse the pressure development in the small calf muscle vein during series of rhythmic muscle contractions A state of maximal exercise vasodilatation was achiebed by two series of induced rhythmic muscle contractions, and the venous pressure development was followed during both these series of contractions A series of 6 successful experiments of this type was performed Also a second series of 2 expts, using the isolated calf muscle preparation proper but carrying out the same measurements gave very similar results During each induced muscle contraction pressure rose markedly in the small calf vein as well as in the femoral vein (Fig 1—3) Whether or not the small vein pressure would subsequently fall below its average prestimulation level depended on the type of nerve stimulation given and thereby on the type of muscle contraction induced If at an impulse frequency of 30—80 per sec the individual impulse trains lasted only 50—100 msec hardly any pressure fall in the small vein could be seen (Fig 1) If however the stimulation trains each lasted for 150 msec or more a very marked reduction in venous pressure would follow each contraction (Fig 2) Similarly with impulse trains lasting long enough *e g* 200 msec the pressure reducing effect of a contraction was very marked with a stimulation frequency of 40—80 per sec whereas a stimulation frequency of 10—20 per sec was usually not enough to produce an equally good pressure lowering effect Under our experimental conditions with maximally dilated vessels, a very good pressure reduction in the superficial muscle calf vein was obtained with stimulation frequencies of 40—60 per sec and with impulse trains lasting 200—300 msec This is a stimulation pattern which should mimic that connected with natural running reasonably well

If the venous pressure was increased beforehand a very marked venous pressure-lowering effect of the muscle pump could be seen (Fig 2) The pressure could thus

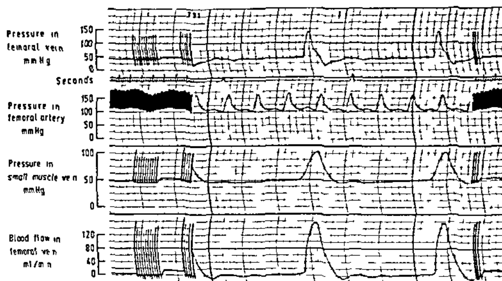


Fig 1 *Effect of rhythmic stimulations of sciatic nerve with 1 impulse train per sec, each train lasting 100 msec on pressure in small calf muscle vein* Single nerve stimulations in the trains carried out at 4 V, for 0 2 msec and with frequency of 60/sec Isolated calf muscle preparation

lowered after these brief phases of contraction

by efficient muscle contractions be reduced from *e g 55 mm Hg to about 25 mm Hg* between contractions Such a pressure reduction must represent a corresponding augmentation in perfusion pressure in the periods between contractions, as long as arterial pressure remains the same The section at high paper speed in Fig 2 shows how well the pressure in the small vein is kept lowered up to the point of the next contraction, despite the [illegible] inflow of blood into the venous system If however, the interval between two contractions was prolonged then the pressure in the small vein increased towards the end of the inter-contraction period indicating a filling up of the venous system (Fig 3) The tracings from Fig 2 and 3 also show how nearly all the blood outflow from the preparation took place during the contractions

In the last series of experiments, with the isolated calf muscle preparation of severed legs, a more quantitative evaluation of muscle pump effect was made possible In particular the effect of the leg being placed in a dependent position could now be analysed 4 successful expts of this type were carried out and the results were rather uniform The pressure in the small muscle vein was not followed in these experiments Typical tracings from such experiments are shown in Fig 4 and 5 Each contraction is accompanied by a spurt of venous outflow from the preparation Especially in the leg down position there is hardly any outflow between contractions (Fig 4) In Fig 5 the blood inflow and the blood outflow-patterns (from consecutive,

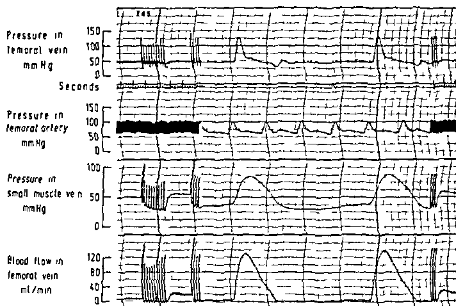


Fig 2 Effect of rhythmic stimulations of sciatic nerve with 1 impulse train per sec, each tra lasting 250 msec, on pressure in small calf muscle vein Single nerve stimulations in the trai carried out at 4 V, for 0 2 msec and with frequency of 60/sec Isolated calf muscle preparatic (right leg) of … reparatio also recorded … ube leadi blood back to … by electr magnetic flow … ubstantial lowered after contraction phases of this duration

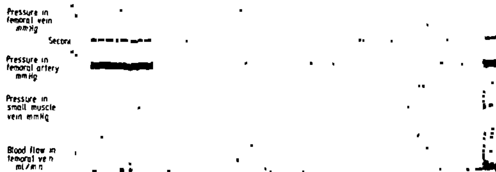
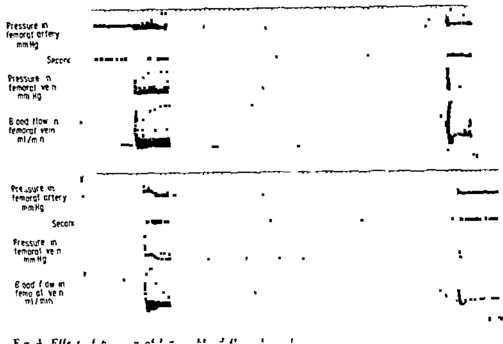


Fig 3 Effect of rhythmic stimulations of sciatic nerve with 1 impulse train per 2 sec each trai … n the train

during these prolonged relaxation phases



tion

Upper set of tracings Leg placed 33 cm (25 mm Hg) below the heart a series of rhythmic contractions is shown followed by a short period of no contractions and "free flow through preparation Note a venous pressure difference of 20—25 mm Hg between contractions as compared with free maximal flow

Lower set of tracings During a series of rhythmic contractions leg is elevated from 33 cm below heart up to heart level Subsequent to period with muscle contractions again a period with no contractions and "free flow Note difference in flow during rhythmic exercise in the two leg positions while "free flow is about the same

identically performed tests in the same preparation) have been superimposed The flow pattern is thus that of virtually no inflow during the contraction phases whereas nearly all the outflow then takes place Between contractions however there is very little outflow whereas inflow is then maximal

The integrated flow values obtained during periods of rhythmic contractions were compared with those seen immediately after the contraction series with the vascular bed still maximally dilated The flow values indicated for Fig 4 are typical and results from two other experiments of this type are given in Table I The net effect of rhythmic contractions in a leg which was level with the body was to reduce the mean flow through the working muscles relative to the situation of free flow through the maximally dilated vascular bed in the post-exercise situation The reduction was however not as great as would be expected when one knows that there

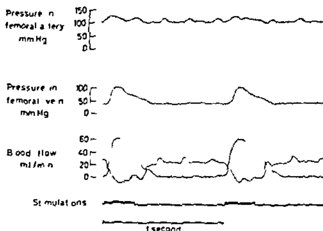


Fig 5 *Relationship between flow pattern in the artery to, and in the vein from, an isolated, rhythmically contracting calf muscle preparation* Tracings from same experiment as Fig 4 Leg in position 33 cm below heart level Sciatic nerve stimulation with 1 impulse train per sec, each train lasting 250 msec Other stimulation parameters 0 2 msec, 4 V, 60 imp/sec Superimposed flow patterns from two consecutive tests shown, in one the probe of electromagnetic flow meter placed on artery, in the other on vein

was almost complete interruption of inflow for about 1/3 of the time, namely during each contraction phase

In the leg down position the mean flow through the rhythmically working muscles was considerably increased, when compared with the corresponding situation in the elevated leg To exemplify from Fig 4 arterial and venous pressures of the limb at heart level position and in the post-exercise period were about 110 and 30 mm Hg respectively and flow during rhythmic exercise 17 3 ml/min For the leg down position *adding about 25 mm Hg to both arterial and venous pressures (which in the* post-exercise period were thus about 135 and 55 mm Hg respectively), flow for the same exercise pattern was 23 6 ml/min implying a gain in flow of 36 % As maximal vasodilatation was at hand this suggests a gain in effective perfusion pressure of about the same magnitude

Moreover in a leg which was kept in the down position, mean flow during rhythmic exercise was as great as—or even greater than—the 'free' flow obtained in the

TABLE I Blood flow through two isolated calf muscle preparations during and just after periods of heavy rhythmic exercise (see Text)

Position of leg		Flow in ml/min	
		Experiment a	Experiment b
At heart level	During exercise	15 6	30 2
	After exercise	18 0	32 0
33 cm below heart level	During exercise	19 8	34 1
	After exercise	20 0	31 0

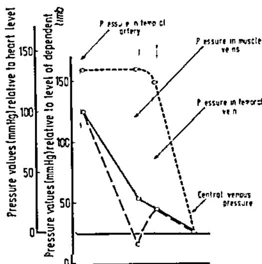


Fig 6 *Schematic demonstration of alterations in pressure in femoral artery, in calf muscle veins and in femoral vein of dependent cat limb during rhythmic exercise* Pressure values from model experiment at maximal flow levels, with isolated cat limb placed 33 cm (25 mm Hg) below heart level (see text) Mean arterial pressure at heart level 100 mg Hg

O———O Mean pressure values in the resting calf

O— —O Mean pressure values between two muscle contractions

O------O Peak pressure values during a muscle contraction

Note that the femoral arterial pressure increases somewhat during muscle contractions (Fig 4), probably as a result of some reflux of blood from compressed muscle vessels

Pressure alterations similar to those illustrated here would be expected to occur *in the dependent limb of a running cat*

post exercise situation of a maximally dilated vascular bed The gain in effective perfusion pressure causing this large flow during rhythmic exercise can again be estimated from data in Fig 4 As is seen from Fig 5 inflow to the vascular bed of the rhythmically contracting muscles takes place only in the intervals between contractions, i e for about 75 % of the whole exercise period The integrated flow value of 23 6 ml/min for this exercise period will thus correspond to an inflow of blood in the intercontraction intervals of about

$$\frac{23 \times 6 \times 100}{75} \text{ ml/min} = 31\ 4 \text{ ml/min}$$

When compared to the 'free' flow of 21 5 ml/min in the immediate post exercise period this represents a gain flow of 9 9 ml/min or about 46 % The corresponding 46 % gain in perfusion pressure must be about

$$\frac{(135 - 55) \times 46}{100} \text{ mm Hg} = 37 \text{ mm Hg}$$

Vascular distension as a result of lowering the leg added in itself very little to the flow at these high levels of transmural pressure In the experiment of Fig 4 free" flow in the post exercise state of maximal vasodilatation was only some 5 % greater in the leg down position than when the leg was level with the heart (21 5 ml/min as against 21 ml/min) Similarly small increments in flow were obtained in other tests where the leg was lowered and transmural pressures thus increased by 25 mm Hg in the period just after a series of rhythmic contractions

Discussion

During heavy rhythmic exercise the resistance vessels in the working skeletal muscles are maximally dilated The main question initiating these experiments was can rhythmic muscle contractions—optimally spaced—in this situation create and maintain local venous pressure reductions in such a way that a substantial gain in effective perfusion pressure is obtained? The answer to this question was an affirmative one Admittedly our experiments are of a model type—and also rather crude ones employing a relatively abnormal type of muscle movements All muscle groups of the calf were initiated to contract simultaneously This does of course not mimic normal walking or running However there is no reason to believe that the venous emptying effect which has here got a first analytical treatment, should be less effective during a more physiological contraction pattern When muscle groups are activated one after the other with normal movements as a consequence one would expect local venous pressure changes to develop in much the same way as here demonstrated during simultaneous contractions If anything the massage effect on the veins in a separate muscle group could even be expected to be more pronounced when its contraction is not counteracted by massive antagonist contraction as in our experiments

The main information obtained from the present tests has been then that the venous vasculature of muscles can be so efficiently emptied during contractions that venous pressure is subsequently considerably reduced even during situations with maximal flow The degree of pressure reduction—and the corresponding gain in effective perfusion pressure—are most marked when the pre contraction transmural pressure is high such as in a dependent limb Under our experimental conditions an optimal venous pressure lowering effect was achieved with stimulus frequencies of 40 –60 per sec and with stimulus trains lasting for 200—300 msec This should not be too far from the pattern employed in the legs of man running or bicycling at a reasonable pace At intense voluntary contraction of the triceps muscle in man impulse frequencies of this order have been observed (Adrian and Bronk 1929) Therefore the stimulation frequency necessary to obtain an optimal effect in our test 40—60 imp/sec does not seem to be unreasonably high in connection with intense activity of phasic skeletal muscles It should also be remembered that our venous pressure measurements had to be carried out in a superficial vein situated between the muscle and the fascia One would expect venous compression to be more marked in deeper muscle veins Perhaps less intense stimulation would suffice to develop efficient emptyings of such veins Our flow studies in dependent isolated limbs seemed to indicate that this was the case there being very efficient pumping during contractions initiated by impulse trains with frequencies of 10—20 imp/sec

For this venous emptying mechanism to play a role by augmenting local flow it is necessary not only to get a significant immediate lowering of post contraction pressure The venous pressure must also be kept reduced for a long enough fraction of the inter-contraction period The relative duration of contraction periods and of inter-contraction periods must here be of critical importance But there is also the

important aspect of the capacity of the rhythmically compressed venous vascular compartment How quickly will the huge inflow into the vessels fill them up—thus raising again their transmural pressure towards the pre-contraction level? In our tests on cat calf muscles with maximal exercise vasodilatation the venous compartment did apparently have sufficient capacity for receiving the huge flow for about 0 5—0 7 sec without then developing a significant rise of local venous pressure (Fig 2 and 3) Venous pressure was well kept down if contractions were induced by in pulse trains lasting 0 2—0 3 sec and being repeated once per sec a pattern of rhythmic exercise which might reasonably well mimic that in the legs of running man If a new contraction did not appear about 0 7 sec after the end of the previous one then the pressure gain was lost and also venous outflow from the preparation would start

The maximal flows seen in our isolated calf muscle preparations of cats averaging 3—3 5 kg in weight were of the order of 20—25 ml/min The muscle group studied weighs about 45 g in a 3 kg cat This equals the previously found maximal blood flow values for this muscle group and for the actual pressure head about 50 ml/min ×100 g of tissue (Folkow and Halicka 1968) One can then calculate the actual amount of blood which the venous part of this vascular bed can receive after having been emptied by a muscle contraction and without becoming distended to such a degree that the lowered transmural pressure is again markedly raised Per 100 g of tissue this amount of blood is 50 ml per 60 sec during 0 6 to 0 8 sec *i e* 0 5 to 0 7 ml

The muscle pump mechanism may thus efficiently increase the average local perfusion pressure thereby increasing local flow through the working muscles even when the vessels are already maximally dilated The effect is particularly marked in situations with high transmural pressures—*e g* in a dependent limb In our experiments total flow through the muscles of a dependent leg was increased by this mechanism (Table I) in spite of the fact that inflow was closed off for about 1/3 of the time This must mean that lower limb muscle groups can be made capable of carrying out more work in the form of rhythmic exercise when in a dependent position than when level with the heart It is known that cardiac output is greater when hard cycling is performed with legs down than with legs level with the body and the explanation for this may lay in the larger flow improving effect of the muscle pump in dependent muscle groups in the former condition Vascular distention *per se* is of only slight importance for flow augmentation in this situation since at these high transmural pressure levels the skeletal muscle resistance vessels are close to their rigid jacket limitation (Folkow and Lofving 1956) This was confirmed by the findings in the present experiments

It appears that the rhythmically working muscles in a limb—and particularly in a dependent one may well deserve the often used designation the peripheral heart As pointed on by Stegall (1966) they provide a large portion of energy for pumping the blood around the circulation during lower limb exercise Furthermore their vasculature—and particularly their veins fill and empty and get their pressure alter

ed in a way resembling the events in the heart ventricles The pressure gradients schematically alter as shown in Fig 6 During a contraction, a "systole", of this peripheral heart,—pressure in the veins increases towards the systemic arterial pressure level Also the local arterial pressure may show a temporary increase (Fig 4), probably as a result of some blood reflux during the strong muscle contraction There is no pressure gradient between arteries and veins and no inflow to the pumping chamber—the veins There is now, however, a large pressure gradient between the muscle veins and the central veins (as also found by Stegall 1966) Blood is thus ejected out of muscle veins with great force and at a high pressure Inbetween contractions there is a lowered pressure in the emptied muscle veins—an increased gradient for flow into them—and an augmented such flow During this filling phase the diastole virtually no blood is flowing centrally, thanks to a negative pressure gradient and competent valves in the ascending venous system

Our experiments have been carried out in a model preparation—and in an animal with short limbs It would be of the greatest interest to investigate the flow values in human lower limbs with a maximally dilated vascular bed, with and without correctly spaced rhythmical work One would expect the mechanism to play a most significant role in the running human Such investigations are now being carried out in this department and the results confirm that a marked gain in regional pressure head and in flow through the working muscles is obtained

One of the authors (B A W) has received a research grant from The Norwegian Research Counci[illegible] which this work was carried out
This st[illegible] een sponsored in part by the Air
Force [illegible] ice of Aerospace Research OAR
United [illegible] a grant from US Public Health
Service [illegible] rch Council (B69 14X-16-05A)
and fr[illegible]

References

Adrian E D and D W Bronk Impulses in motor nerve fibres *J Physiol* (Lond) 1929 *67* 119 151

Allwood M J The effect of an increased local pressure gradient on blood flow in the foot *Clin Sci* 1957 *16* 231—239

Barcroft H and A C Dornhurst Demonstration of the muscle pump in the human leg *J Physiol* (Lond) 1949 *108* 39P

Folkow B P Gaskell and B A Waaler Blood flow through muscles during heavy rhythmic exercise *Acta physiol scand* 1969 *76* 22A—23A

Folkow B and H D Hallicka A comparison between red and white muscle with respect to blood supply capillary surface area and oxygen uptake *Microvascular Res* 1968 *1* 1—14

Folkow B and B Lofving The distensibility of the systemic resistance vessels *Acta physiol scand* 1956 *38* 37 52

Hoyensgård I C and H Sturup Static and dynamic pressures in superficial and deep veins in the lower extremity of man *Acta physiol scand* 1952 *27* 49—67

Kjellmer I The effect of exercise on the vascular bed of sceletal muscle *Acta physiol scand* 1964 *62* 18—30

Ludbrook J, Functional aspects of the veins of the leg *Amer Heart J* 1962 *64* 706—713

[illegible] ankle in man

[illegible] 180—190

[illegible] exercise as an

Acta physiol. scand. 1970. 80. 73—78

From the Institute of Physiology, University of Lund, Sweden

Distribution of Choline Acetyltransferase in the Hearts of Mammals

By

JORGEN EKSTROM

Received 29 January 1970

Abstract

EKSTROM, J *Distribution of choline acetyltransferase in the hearts of mammals* Acta physiol scand 1970 *80* 73—78

Choline acetyltransferase activity was estimated in hearts of dogs cats, rabbits and rats The ventricles, which form about 90 per cent of the fresh weight of the hearts are responsible for about two thirds of the total enzyme activity of the heart in dogs cats and rats and in rabbits for about half the total activity of the heart The concentration of the enzyme is higher in the atria than in the ventricles In dogs the apex of the ventricles showed the same enzyme concentration as the basal parts, in cats and rabbits it was somewhat lower in the apex than at the base The findings are discussed in relation to the controversial question of a parasympathetic innervation of the mammalian heart ventricles

---

Textbooks of Physiology generally state that in mammals the parasympathetic cardiac nerves supply the atria but not at all the ventricles or at the most their basal parts only A great number of observations, anatomical and physiological, support this view Particularly in recent years, however, this opinion has been questioned and evidence has been produced to show that the parasympathetic innervation of the ventricles is more extensive than assumed earlier The literature was reviewed by DeGeest *et al* (1965), since then, several papers have appeared which report observations suggesting that the ventricles of mammals receive parasympathetic fibres (Cooper *et al* 1967, Jacobowitz, Cooper and Barner 1967, Ehinger *et al* [illegible], Cronin Armour and Randall 1969, Ferrante and Opdyke 1969)

One way of approach to this problem has been to study the occurrence and release in the ventricles of the parasympathetic transmitter acetylcholine or the presence of enzymes engaged in its metabolism Early experiments demonstrated acetylcholine in extracts both of atria and ventricles of some mammals (Engelhart 1930, Chang and Gaddum 1933), and this was confirmed in later investigations ([illegible]) In the perfusate of the isolated heart of the rabbit acetylcholine was found to appear even when the atria had been removed (Day 1956) Cholinesterase activity was demonstrated both in atria and ventricles but mainly of the unspecific [illegible] acetylcholinesterase seems to be related especially to the conductive [illegible]

heart (Antopol, Glaubach and Glick 1939, Gowier *et al* 1953 Mommaerts Khairallah and Dickens 1953)

Synthesis of acetylcholine in the different parts of the heart is of particular interest in this connexion In the turtle heart choline acetyltransferase activity was demonstrated not only in the sinus and atria, but in the ventricles also (Mazel and Holland 1958) In mammals, however, the activity was found to be confined to the atria (Comline 1946, Ebashi 1954) This would seem to support the concept that the mammalian ventricles lack a parasympathetic innervation. In view of the fact that improved methods for demonstration of choline acetyltransferase activity are now available it was, however, thought desirable to reinvestigate the question of the distribution of the enzyme in various parts of the hearts of mammals

Methods

Hearts were removed from 5 cats 5 dogs 5 rabbits and 25 rats They were carefully cleaned the visceral pericardium attached to the myocardium being left intact and divided into two parts atria and ventricles In the case of the rats 5 hearts were pooled, but otherwise each heart was treated separately The ventricles of the cats, dogs and rabbits were further divided into a basal part corresponding to about 40 per cent of the ventricles and the remaining apical part In cats and rabbits the whole of these two parts were used for the experiments From the big ventricles of dog hearts only pieces were collected from base and apex but care was taken to secure these both from the walls of the two ventricles and from the septum In an additional series of experiments ventricles from 3 cats 3 rabbits and 15 (3×5) rats were divided into 3 parts left ventricle right ventricle interventricular septum

All the preparations were weighed and acetone dried powders were made The choline acetyltransferase activity of these powders was estimated using the mehod of Hebb as described by Nordenfelt (1963) The acetylcholine synthesized during 1 hr of incubation was assayed on the frog rectus preparation according to Feldberg (1950) Enzyme activity was expressed as μg of acetylcholine chloride produced during 1 hr either per gram acetone powder per whole heart or per part of the heart (atria ventricles base apex left or right ventricle, interventricular septum

Results

The distribution of choline acetyltransferase in the various parts of the heart of the cat the dog the rabbit and the rat is shown in Table I II III and IV, respectively

The whole heart When comparing the four species the total activity of the enzyme

TABLE I Choline acetyltransferase activity in the heart of the cat (mean ±S E M n 5)

Tissue	Fresh weight per cent of heart weight	Enzyme activity		
		Total activity μg ACh hr	Activity in per cent of that of whole heart	Concentration μg ACh hr g acetone powder
Whole heart (fresh weight 7 1±0 74 g)		160±20		113±9 0
Atria	12 9 0 73	59±8 0	37±1 0	340±31
Ventricles	87 1 0 73	100±13 2	63±1 0	82±6 8
Base	37 ±1 3	50±6 3	31±1 1	94±6 0
Apex	50 ±1 1	52±7 4	32±1 4	72±7 6

TABLE II Choline acetyltransferase activity in the heart of the dog (mean ± S E M , n = 5)

Tissue	Fresh weight per cent of heart weight	Enzyme activity		
		Total activity µg ACh/hr	Activity in per cent of that of whole heart	Concentration µg ACh hr/g acetone powder
Whole heart (fresh weight 53±2 8 g)		1150±54		110±11
Atria	10 6±0 25	380±41	33±2 6	400±63
Ventricles	89 4±0 25	770±38	67±2 5	83±7 4
Base	33 5±0 65	300±25	26±1 3	90±10
Apex	56 ±1 1	480±29	42±2 9	80±6 6

in the heart is found to be higher the larger the heart Thus it is about 7 times higher in the dogs than in the cats, the fresh weight of the heart is roughly 7 times larger in the dogs than in the cats In rabbits the total activity is about half of that of the cats, the hearts of the rabbits are smaller than those of the cats but not as little as half their sizes In rats finally the activity is 4—5 times smaller than in cats but when the weight of the hearts is taken into account this is a comparatively high activity the hearts of the cats being about 9 times bigger than those of the rats

When the concentration of the enzyme is expressed as activity per gram acetone powder, as is usually done it is found to be the same in dogs and cats (110 and 113 µg acetylcholine per hour), somewhat smaller in rabbits (76) but considerably higher in rats (198)

Atria and ventricles In all the species the fresh weight of the atria was about 10 per cent and the ventricles about 90 per cent of that of the whole heart As to the enzyme activity, however, the contribution of the atria was much larger about one third of the total activity of the heart in dogs, cats and rats (dogs 33 cats 37 and rats 29 per cent), and in rabbits it was even larger (53 per cent)

TABLE III Choline acetyltransferase activity in the heart of the rabbit (mean ± S E M , n = 5)

Tissue	Fresh weight per cent of heart weight	Enzyme activity		
		Total activity µg ACh/hr	Activity in per cent of that of whole heart	Concentration µg ACh/hr/g acetone powder
Whole heart (fresh weight 6±1 0 g)		80±18		76±9 9
Atria	14 4±0 83	39±6 7	53±3 5	320±39
Ventricles	85 6±0 83	40±12	47±3 5	41±6 2
Base	36 ±1 1	21±5 7	26±1 3	54±8 2
Apex	50 ±1 4	18±6 5	22±2 7	32±4 9

TABLE IV Choline acetyltransferase activity in the heart of the rat (mean ±S E M , n = 5)

Tissue	Fresh weight, per cent of heart weight	Enzyme activity		
		Total activity µg ACh/hr	Activity in per cent of that of whole heart	Concentration µg ACh hr/g acetone powder
Whole hearts (5 pooled, fresh weight 4 2±0 17 g)		180±12		198±8 7
Atria (5 pooled)	9 7±0 13	53±2 4	29±1 4	740±27
Ventricles (5 pooled)	90 3±0 13	130±10	71±1 4	152±8 0

The concentration of choline acetyltransferase (in µg acetylcholine /hr/g acetone powder) in the atria was of the same order of magnitude in dogs, cats and rabbits, it was higher in rats In the ventricles it was considerably smaller than in the atria 1/4—1/5 of that of the atria in dogs, cas and rats and about 1/8 in rabbits

Base and apex of ventricles These were compared in dogs, cats and rabbits The ventricles were divided in such a way that base' corresponded to about 40 per cent and apex to about 60 per cent of the ventricles fresh weight (dog 38±1 1 and 62±1 1 cat 43±1 3 and 57±1 3 rabbit 42±1 4 and 58±1 4 per cent) In dogs the enzyme activity showed the same distribution *i e* about 40 per cent of the total activity was found in the base and 60 per cent in the apex The concentration of the enzyme was in other words the same in base and apex of dogs (fresh weight), and this was found to be true when calculated for acetone powder also In cats and rabbits the total activity of the enzyme was about the same in the two parts of the ventricles in spite of the fact that here also the base comprised about 40 and the apex 60 per cent of the ven ricles Correspondingly the enzyme concentration was somewhat higher in the base than in the apex in these two species and this applies to the acetone powders as well (calculated per gram acetone powder 0 05<P<0 1 for cats and P<0 05 for rabbits

TABLE V Choline [illegible]

Animal		Left ventricle	Septum	Right ventricle
Cat	weight	49 5 0 75	27 4±0 46	23 ±1 1
	activity	48 3 0	23 1 5	29 ±2 2
Rabbit	weight	43 ±2 1	32 9±0 66	24 ±2 1
	activity	33 1 0 72	34 ±3 6	33 ±4 2
Rat	weight	46 6 0 70	30 ±1 1	23 0±0 51
	activity	38 0±0 92	28 7±0 61	33 ±1 3

Septum and walls of ventricles The results of these experiments on cats rabbits and rats are shown in Table V For each separate part the table gives the total enzyme activity and the fresh weight in per cent of those of the whole ventricular muscle Choline acetyltransferase activity could be demonstrated in the three parts of the ventricles in these species It can be calculated from the figures that the concentration of the enzyme is highest in the wall of the right ventricle

Discussion

The experiments show that using the method of Catherine Hebb it is possible to demonstrate choline acetyltransferase activity in the heart of a number of mammals Milton (1959) detected a concentration of the enzyme in the atria of rabbits which is similar to that found in the present work

The main finding of this investigation is that there is a choline acetyltransferase activity not only in the atria but in the ventricles also and that this applies both to the basal and the apical part of the ventricles and also to the interventricular septum and the outer walls of the ventricles It is tempting to infer that this indicates that there is a supply of parasympathetic fibres to the various parts of the ventricles in mammals It may be pointed out that the concentration of the enzyme is higher in the atria than in the ventricles to some extent at least this might be due to the fact that nerves for the ventricles have to pass the atria The oft-quoted observation that a nerve free organ like the placenta can synthesize acetylcholine (Comline 1946 Hebb and Ratkovic 1962) may serve to emphasize that choline acetyltransferase activity is no definite proof of the presence of cholinergic fibres At any rate, the present experiments show that earlier investigations reporting that choline acetyltransferase occurs in the atria but not in the ventricles cannot be used in support of the view that the ventricles receive no parasympathetic fibres

The findings regarding the distribution of choline acetyltransferase in the different parts of the heart are on the whole, in agreement with previous observations on acetylcholine and cholinesterase The concentration of acetylcholine extracted from the atria is higher than that of the ventricles (Engelhart 1930 Chang and Gaddum 1933, Rothschuh 1954) Likewise the concentration of cholinesterase is higher in atria than in ventricles (Antopol *et al* 1939) The concentration of acetylcholine is about twice as high in the outer wall of the right as in that of the left ventricle of the rat (Rothschuh 1954) the same holds true for the synthesizing enzyme (Table V)

References

ANTOPOL W S GLAUBACH and D GLICK Choline esterase activity in various portions of the rabbit heart *Proc Soc exp Biol* (N Y) 1939 *42* 280—282

CHANG H C and J H GADDUM Choline esters in tissue extracts *J Physiol* (Lond) 1933 *79* 255—285

COMLINE R S Synthesis of acetylcholine by non nervous tissue *J Physiol* (Lond) 1946 *105* 6—7 P

COOPER T E F HIRSCH G C KAISER and H B BARNER Topography of cardiac innerva

tion loci and distribution of points of sympathetic and parasympathetic entry *Cl n Res* 1967 *15* 199

CRONIN R J A ARMOUR and W C RANDALL Function of the in situ papillary muscle in the canine left ventricle *Circulat Res* 1969 *25* 67—75

DALE H The release of substances like acetylcholine and adrenaline by the isolated rabbit heart *J Physiol* (Lond) 1956 *134* 558—568

DEGEEST H M N LEVY H ZIESKE and R I LIPMAN Depression of ventricular contraction by stimulation of the vagus nerves *Circulat Res* 1965 *17* 222—235

EBASHI S Studies on choline acetylase (2) *Jap J Pharmacol* 1954 *4* 32—40

EHINGER B B FALCK H PERSSON and B SPORRONG Adrenergic and cholinesterase-containing neurons of the heart *Histochemie* 1968 *16* 197—205

ENGELHART E Ueber humorale Uebertragbarkeit der Herznervenwirkung XIII Mitteilung Die Vagusstoffverteilung auf Vorhof und Kammer bei Frosch und Sauger *Pflugers Arch ges Physiol* 1930 *225* 21—22

FELDBERG W Synthesis of acetylcholine (choline acetylase) *Meth med Res* 1950 *3* 95—106

FERRANTE F L and D F OPDYKE Mammalian ventricular function during submersion asphyxia *J appl Physiol* 1969 *26* 561—570

GOVIER WM M W A FREYBURGER A J GIBBONS B G HOWES and E SMITS The relation of the choline cycle to cardiac decompensation acetylcholine metabolism in the dog heart lung preparation *Amer Heart J* 1953 *45* 129—143

HEBB C O and D RATKOVIĆ Choline acetylase in the placenta of man and other species *J Physiol* (Lond) 1962 *163* 307—313

JACOBOWITZ D T COOPER and H B BARNER Histochemical and chemical studies of the localization of adrenergic and cholinergic nerves in normal and denervated cat hearts *Circulat Res* 1967 *20* 289—298

MAZEL P and W C HOLLAND Acetylcholine and electrolyte metabolism in the various chambers of the frog and turtle heart *Circulat Res* 1958 *6* 684—688

MILTON A S The enzyme systems concerned with acetylcholine synthesis in the atria of the rabbit heart *J Physiol* (Lond) 1959 *149* 462—470

MOMMAERTS W F H M P A KHAIRALLAH and M F DICKENS Acetylcholinesterase in the conductive tissue of the heart *Circulat Res* 1953 *1* 460—465

NORDENFELT I Choline acetylase in normal and denervated salivary glands *Quart J exp Physiol* 1963 *48* 67—79

ROTHSCHUH K E Das herzmuskeleigene Acetylcholin II Mitteilung Der normale Acetylcholin-Gehalt der Vorhofs und Kammermuskulatur beim Frosch und bei der Ratte *Pflugers Arch ges Physiol* 1954 *258* 481—488

Acta physiol scand. 1970. 80 79—92

From the Institute of Pharmacology University of Uppsala Sweden

Time Relations of Degeneration Mydriasis and Degeneration Vasoconstriction in the Rabbit Ear after Sympathetic Denervation. Effect of Bretylium.

By

ERNST H BÁRÁNY and GIORA TREISTER[1]

Received 2 February 1970

Abstract

BÁRÁNY, E H and G TREISTER *Time relations of degeneration mydriasis and degeneration vasoconstriction in the rabbit ear after sympathetic denervation Effect of bretylium* Acta physiol scand 1970 80 79—92

Removal of the superior cervical ganglion in rabbits causes homolateral mydriasis starting about [illegible] starts [illegible] tive to [illegible] ed by [illegible] tip of [illegible] -3 hrs would have been detected The conclusion is that the time between operation and transmitter release from the degenerating terminals is longer than is needed for the message of the interruption of the axon to reach the terminals Time differences between degeneration release of transmitter in different organs may be partly due to intrinsic differences between the populations of terminals

Earlier studies on the nictitating membrane of the cat by the Trendelenburg group (Smith *et al* 1966 Langer 1966 and van Orden *et al* 1967) on the periorbital muscle of the rat (Lundberg 1969) on the vessels of the rabbit ear (Emmelin and Ohlin 1969) and on the rabbit pupil (Treister and Bárány 1970a) have shown that the transient spontaneous contraction of a sympathetically innervated smooth muscle following extirpation of the sympathetic ganglion is due to a leakage of transmitter from the degenerating nerve terminals The degeneration release and the resulting muscle contraction always start at an interval after the denervation The interval ranges between 8 and 26 hrs depending on the experimental animal organ or method (Kirpekar *et al* 1962 Benmiloud and Euler 1963 Malmfors and Sachs 1965 and the papers quoted above)

[1] Work done during leave of absence from Tel Hashomer Hospital Tel Aviv University Medical School Israel

What causes this interval? Time is needed for 'the news of the missing cell body to reach the nerve terminals It is reasonable to assume that this time depends on the distance from the point of axotomy to the terminals The latency between interruption of the axon and start of the degeneration release should therefore depend on the length of the nerve stump But there may also be a latency period between the arrival of the information and the start of the degeneration

In the cholinergic system Emmelin (1965, 1966, 1968) has already demonstrated such a dependence on the distance between the lesion and the terminal The present paper is concerned with the adrenergic system and presents a study in the conscious rabbit of two degeneration phenomena after cervical ganglionectomy—the degeneration vasoconstriction of the ear vessels and the degeneration mydriasis The length of the sympathetic nerves to the base of the ear must differ from those to the tip and at least the latter must be longer than those to the eye It was of interest to compare the time relations of the degeneration release of transmitter in these three locations and to see if the findings of Emmelin in the cholinergic system have a counterpart in the adrenergic system

Earlier studies have shown that the adrenergic neuron blocker bretylium delays the denervation phenomena (Lundberg 1969, Treister and Bárány 1970b) by postponing the leakage of transmitter out of the degenerating nerve terminals (Benmiloud and Euler 1963 Malmfors and Sachs 1965) We compared the effect of bretylium on the degeneration vasoconstriction and on the degeneration mydriasis

Material and methods

22 pigmented and 4 albino rabbits of both sexes weighing 1 7—3 5 kg were used Care was taken to select animals with healthy ears Commercial food pellets and water were provided ad lib Left preganglionic sympathotomy (decentralization) and right cervical ganglionectomy (denervation) were performed under pentobarbital anesthesia 30—40 mg/kg i p The ears were not used The technique was that employed by Sears and Bárány (1960) Three rabbits underwent bilateral adrenalectomy 24 hrs prior to the denervation and decentralization The technique used was that of Zak Good and Good (1957) These animals were given 25 mg cortisone acetate and 5 mg DOCA i m at time of operation They were kept on 1 % NaCl in the drinking water

Technique and measurement

All the observations were made on conscious animals handled with care so as not to be irritated Both phenomena—the mydriasis and the vasoconstriction of the ear vessels were measured simultaneously on the same animals The technique of measurement of the pupillary size has been described (Treister and Bárány 1970a) Briefly the rabbit was supported on wire mesh and immobilized by a nylon net The lens was made to fluoresce by ultraviolet light The horizontal diameter of the pupil was measured in darkness by a spring bow caliper with fluorescent tips which were adjusted precisely to the border between the dark edge of the iris and the fluorescent lens The distance between the tips was then measured with a ruler with interpolation to 0 1 mm The four albino rabbits were measured by the same caliper under ordinary artificial room illumination

Measurements were repeated at least hourly except for 4 hrs of night sleep Each pupillary size value was the mean of at least 4 readings in rapid succession The single readings varied at most by ±0 1 mm

The vasoconstriction of the denervated ear was followed by measurement of the temperature difference between corresponding points as a rule only at the tip on the denervated and the decentralized ear Fig 1 at a room temperature of 22 26° C In some animals bilateral ganglionectomy was performed and the temperature difference recorded between the ambient air and two points on each ear tip and base

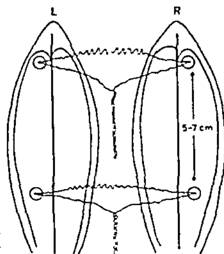


Fig 1. The place and arrangement of the thermojunctions on the tip and base of the ears Dorsal aspect

After careful removal of the hairs with scissors, thermojunctions (copper-constantan) were [illegible]
Hg thermometer

Calculations

The design of the calculation and the graphical representation of the phenomena were those employed earlier (Treister and Barány 1970a) Each value represents the difference (D) between the denervated and the decentralized sides. The individual curves were constructed by plotting D values against time after denervation Fig 2 indicates the measurements that were taken

Drugs

Doses mentioned in the text refer to the salts All solutions contained 0 9 % NaCl

Bretylium tosylate (Dr A. F Green, the Wellcome Research Laboratories, Beckenham, Kent England) Ten mg/ml was used

Hexamethonium bromide was used at 20 mg/ml and

Mecamylamine hydrochloride at 2 mg/ml

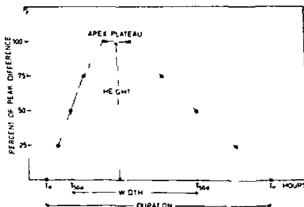


Fig 2 Graphical representation of the main parameters of the degeneration mydriasis and the degeneration vasoconstriction

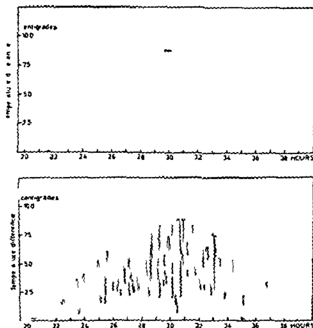


Fig 3 Semi schematic diagram Two individual curves of the temperature change during the degeneration vasoconstriction The curves demonstrate two different types of waves

The broken line which connects the tips of the waves shows how the curves were evaluated

Results

The results concerning the degeneration mydriasis which are used here have been reported and discussed earlier (Treister and Barany 1970 a b)

While the transient degeneration mydriasis is a very consistent phenomenon and appears in each animal after denervation the transient degeneration vasoconstriction of the ear vessels after denervation is less consistent

1 The vasoconstriction did not appear at all in one animal and as 1—2 hr waves that could not be evaluated in 5 animals out of 26 These animals were excluded

2 In several cases there was a time difference between the start of the temperature change and the visually observed vasoconstriction either the one or the other appeared 1—3 hrs later We used the temperature data only

3 It was very difficult to define the end point of the vasoconstriction *The temperature difference between the two ears decreased but did not disappear* Sometimes more than 30 % *of the maximum temperatur difference remained* This difference could be completely eliminated by hexamethonium 10 mg/kg i m Bilateral adrenalectomy *failed to prevent it In contrast the residual mydriasis* was always less than 25 % of the maximum and was not influenced by hexamethonium or by bilateral adrenalectomy

4) The individual temperature curves were not smooth they consisted of waves of about 15—30 min duration The amplitudes of these waves varied from animal to animal ranging between 1° C and 10° C (Fig 3) The smaller waves tended to be longer The amplitude of the waves was much smaller at the start of the degenera-

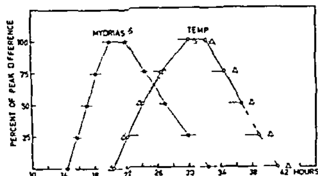


Fig 4 Mean curves of the degeneration mydriasis and the temperature change (representing the degeneration vasoconstriction) Means±S E M n=8 The broken line represents the temperature change measured in a separate group of four undisturbed animals

tion vasoconstriction It was also small in the three adrenalectomized rabbits This can have been a coincidence

The degeneration mydriasis and the degeneration vasoconstriction in untreated animals (Group I)

Fig 4 and Table I show the main parameters concerning these two phenomena The mydriasis starts around 14 5 hrs after denervation (Table I a) while the temperature change starts about 6 hrs later (Table I b) The ascending limb of the temperature difference curve is less steep than that of the mydriasis ($P<0\ 05$) (Table I a b c and Fig 4) Other differences were not statistically significant

The degeneration vasoconstriction in undisturbed animals (Group II)

The denervated ear becomes very sensitive to circulating catecholamines resulting in a considerable vasoconstriction when the rabbits are even slightly irritated Therefore a group of 4 animals were operated late in the evening (9—11 p m) so that the temperature change was expected for the next evening and night The rabbits were fixed on their wire net and the thermojunctions were glued on in the afternoon before the vasoconstriction was expected From this time on nobody was present in the laboratory

Fig 4 demonstrates that the mean temperature change curve of this group was very similar to the mean curve of the previous group

The effect of bretylium given prior to the degeneration mydriasis (Group III)

In this group of 4 animals bretylium 10 mg/kg was injected i m at the time of operation and 8 hrs later Table I (d e) summarizes the main parameters of the bretylium effect

The mydriasis was delayed by 3—5 5 hrs depending on whether one considers T or T_{50a} (Table I h) and the temperature change by about 4 5 hrs (Table I i) The delayed mydriasis in the treated group was longer (Table I h) than the mydriasis in the untreated group The duration width and height of the temperature change were similar in both groups (Table I i)

TABLE I The main parameters of the degeneration mydriasis and the degeneration vasoconstriction in a bretylium treated group and an untreated group. Bretylium 10 mg/kg was injected i.m. at the time of operation and 8 hrs later. Means ± S.E.M. The temperature change is a measure of vasoconstriction

	T_0 hours	$T_{50\%}$ hours	Width hours	Duration hours	Height or Maximum	Inverse slope a[1]	Inverse slope d[2]
(a) Mydriasis untreated group (I) n = 8	14.51 ±0.21	17.05 ±0.66	9.93 ±0.52	18.01 ±0.89	2.78[3] ±0.26	1.24 ±0.21	2.53 ±0.43
(b) Temperature change untreated group (I) n = 8	20.31 ±1.26	24.19 ±1.11	12.50 ±1.92	20.85 ±2.07	8.35[4] ±0.70	2.37 ±0.34	2.21 ±0.32
(c) Difference (b)—(a)	5.83 ±1.30	7.14 ±1.29	2.55 ±1.87	2.83 ±2.14	not compar-	1.13 ±0.51	—0.32 ±0.49
Significance	P<0.001	P<0.001	n.s.	n.s.	able	P<0.05	n.s.
(d) Mydriasis treated group (III) n = 4	17.65 ±0.33	22.56 ±0.27	12.03 ±0.32	23.33 ±1.40	2.89[3] ±0.33	1.92 ±0.28	3.20 ±0.34
(e) Temperature change treated group (III) n = 4	25.02 ±0.97	28.50 ±0.78	12.48 ±1.55	20.79 ±1.92	8.56[4] ±0.62	2.10 ±0.35	2.52 ±0.40
(f) Difference (e)—(d)	7.37 ±1.02	5.94 ±0.82	0.45 ±1.39	—2.54 ±2.16	not compar	0.18 ±0.45	—0.68 ±0.53
Significance	P<0.001	P<0.001	n.s.	n.s.	able	n.s.	n.s.
(g) Difference (f)—(c)	1.99 ±1.64	—2.20 ±1.52	—2.10 ±2.33	0.29 ±3.01	not compar-	—0.95 ±0.67	—1.00 ±0.70
Significance	n.s.	n.s.	n.s.	n.s.	able	n.s.	n.s.
(h) Difference (d)—(a)	3.14 ±0.41	5.51 ±0.94	2.10 ±0.84	5.32 ±1.74	0.11 ±0.40	0.68 ±0.35	0.67 ±0.71
Significance	P<0.001	P<0.001	P<0.05	P<0.05	n.s.	n.s.	n.s.
(i) Difference (e)—(b)	4.71 ±1.59	4.31 ±1.36	—0.02 ±2.34	—0.06 ±2.82	0.21 ±0.92	—0.27 ±0.56	0.31 ±0.50
Significance	P<0.05	P<0.01	n.s.	n.s.	n.s.	n.s.	n.s.

[1] inverse slope of the ascending limb (T_{75a}—T_{25a})
[2] inverse slope of the descending limb (T_{25d}—T_{75d})
[3] mm
[4] centigrades

Table I g shows that on the whole there was no statistically significant difference between the effect of bretylium on the degeneration mydriasis and its effect on the degeneration vasoconstriction.

The effect of a single injection of bretylium 10 mg/kg given during the degeneration mydriasis 15.5—17.0 hrs after denervation (group II)

In 4 rabbits bretylium was given 3 hrs after the start of the mydriasis but before the start of the vasoconstriction. The effect on the pupil of bretylium injected during the degeneration mydriasis has been discussed earlier (Treister and Bárány 1970b). Briefly, about 15 min after the injection the denervated pupil starts to dilate fastly

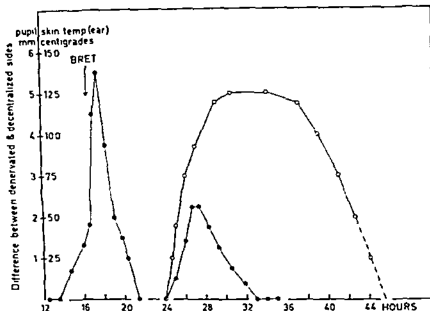


TABLE II Starting times (T_0 and $T_{50\%}$) of the degeneration vasoconstriction and mydriasis in the various groups There were 8 animals in group I and 4 in the others Values are hours after operation, means ± SEM

Group I Untreated animals

Group III Bretylium 10 mg/kg i m given prior to the degeneration mydriasis at the time of operation and 8 hrs later

Group IV One single injection of bretylium 10 mg/kg given during the degeneration mydriasis, 15 5—17 0 hrs after denervation

Group V Two injections of bretylium 10 mg/kg given at the time of denervation and 10 hrs later, and a third injection 4 mg/kg given 19—21 5 hrs after denervation (just before the expected start of the normal degeneration vasoconstriction)

	T_0				$T_{50\%}$	
	Group I	Group III	Group IV	Group V	Group IV	Group V
(a) Vasoconstriction	20 31 ±1 26	25 02 ±0 97	24 51 ±0 84	29 53 ±1 01	28 02 ±0 79	32 20 ±0 96
(b) Mydriasis	14 51 ±0 21	17 65 ±0 33	23 42[1] ±0 35	27 48[1] ±0 42	25.24[1] ±0 36	29 12[1] ±0 39
Significance	P<0 001	P<0 001	n s	n s	P<0 05	P<0 05

[1] Second wave of the degeneration mydriasis when bretylium was given during the phenomenon

(the sympathomimetic effect) and 2.5—4 hrs later returns to its premydriatic value or somewhat (0.5 mm) above it. Around 7 hrs after the injections of bretylium and 23.42 ± 0.35 after the denervation a second wave of mydriasis starts and continues for 7—9 hrs. Vasoconstriction comes as a single wave. Fig. 5 shows a typical experiment where all this is seen.

Table II summarizes the main starting times of the various groups. It shows that the starting times of the temperature change in group III and IV are very similar. Hence, the delay of the phenomenon caused by a single injection of bretylium at 15.5—17.0 hrs after the denervation (group IV) was very similar to the delay caused by 2 injections of bretylium at the time of denervation and 8 hrs later (group III). The duration and the height of the temperature change in these groups were also similar.

In group IV, the starting time of the temperature change is close to the starting time of the second wave of the mydriasis. Using the $T_{5/4}$ values, however, the difference between the two phenomena is probably significant (Table II).

The effect of 2 injections of bretylium 10 mg/kg given at time of denervation and 10 hrs later and a third injection 4 mg/kg given 19—21.5 hrs after denervation (just before the expected start of the normal degeneration vasoconstriction) (Group I)

As described in detail (Treister and Bárány 1970b) the first two injections of bretylium delayed the mydriasis in this group of 4 animals up to around 17.5 hrs after denervation. Two to three hrs after the start of this delayed mydriasis, the third injection was given causing a small sympathomimetic effect which ended in a trough. As in the previous group this trough split the degeneration mydriasis into 2 components. The second component started about 7 hrs after the injection and 27.48 ± 0.42 after the denervation (Table II) and continued for about 14 hrs. Fig. 6 shows a typical experiment.

The temperature change in this group was delayed until 29.53 ± 1.01 hrs after denervation (Table II). There was only a single wave of vasoconstriction. The delay exceeding that of the previous group could be caused only by the late third injection.

The duration, width and height of the phenomenon were similar to those of the previous group. Here, again, there is rather close agreement between the start of the temperature change and the second wave of the mydriasis (Table II).

The $T_{5/4}$ values of the mydriasis and the temperature change again show a larger, probably significant, difference.

The time relations of the degeneration vasoconstriction measured at the tip and the base of the same ear. Group VI

This experiment was intended to show a difference in degeneration time depending on the length of the nerve stump. The basic assumption was that the adrenergic nerve terminals in the base and in the tip of the ear are virtually equal and that the duration of their degeneration release would be equal too. One cannot expect to

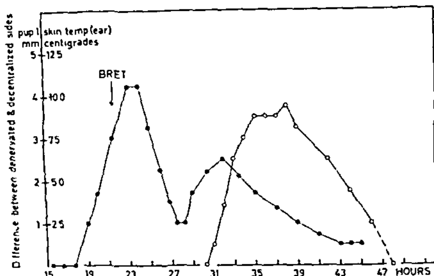


Fig 6 Effect of bretylium on pupil and skin temperature of the ear (representing vasoconstriction) in one experiment of group V Bretylium 4 mg/kg was injected 21 5 hrs after denervation, a few hrs after the start of a degeneration mydriasis already delayed by previous injections of the drug Pupil—filled circles Temperature—open circles Ordinates difference between denervated and decentralized sides

measure differences between tip and base in starting time of the vasoconstriction because when the central artery at the base of the ear contracts the whole ear becomes cold But if the vasoconstriction starts earlier at the base of the ear it has to end earlier than at the tip The base would be warm again while the tip would still be cold This we thought should be possible to see

Two kinds of experiments were made In some the temperature difference between the ears was recorded at the tip as well as at the base (Fig 1) One side was denervated, the other decentralized In other animals the ganglion was removed on both sides and the differences between the room temperature (around 24° C) and the tip and the base of each ear were separately recorded The distance between the thermojunctions at the tip and at the base was 5—7 cm

Since we were interested in the end of the phenomenon the animals were left in their cages after operation and recording started only 27—30 hrs after denervation Since we had no value for the temperature before the onset of vasoconstriction we used as zero line the highest temperature that could be recorded after complete elimination of vasoconstriction by 1—2 mg/kg mecamylamine i m 45—48 hrs after denervation Later injections did not give higher temperature

It turned out to be impossible to define the end of the vasoconstriction by simple observation because the ear tended to stay cold An attempt was made to eliminate circulating catecholamines by blocking release from the adrenal medullae and chromaffin cells by repeated injections of hexamethonium 10 mg/kg Unfortunately

(the sympathomimetic effect) and 2 5—4 hrs later returns to its premydriatic value or somewhat (0 5 mm) above it Around 7 hrs after the injections of bretylium and 23 42 ± 0 35 after the denervation a second wave of mydriasis starts and continues for 7—9 hrs Vasoconstriction comes as a single wave Fig 5 shows a typical experiment where all this is seen

Table II summarizes the main starting times of the various groups It shows that the starting times of the temperature change in group III and IV are very similar Hence the delay of the phenomenon caused by a single injection of bretylium at 15 5—17 0 hrs after the denervation (group IV) was very similar to the delay caused by 2 injections of bretylium at the time of denervation and 8 hrs later (group III) The duration and the height of the temperature change in these groups were also similar

In group IV the starting time of the temperature change is close to the starting time of the second wave of the mydriasis Using the T_{50} values however the difference between the two phenomena is probably significant (Table II)

The effect of 2 injections of bretylium 10 mg/kg given at time of denervation and 10 hrs later and a third injection 4 mg/kg given 19—21 5 hrs after denervation (just before the expected start of the normal degeneration vasoconstriction) (Group V)

As described in detail (Treister and Barany 1970b) the first two injections of bretylium delayed the mydriasis in this group of 4 animals up to around 17 5 hrs after denervation Two to three hrs after the start of this delayed mydriasis the third injection was given causing a small sympathomimetic effect which ended in a trough As in the previous group this trough split the degeneration mydriasis into 2 components The second component started about 7 hrs after the injection and 27 48 ± 0 42 after the denervation (Table II) and continued for about 14 hrs Fig 6 shows a typical experiment

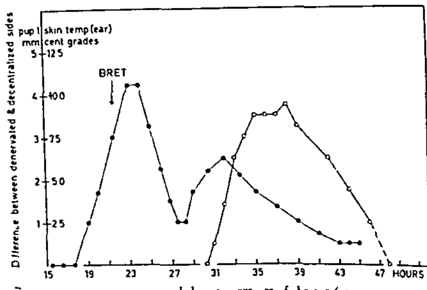
The temperature change in this group was delayed until 29 53 ± 1 01 hrs after denervation (Table II There was only a single wave of vasoconstriction The delay exceeding that of the previous group could be caused only by the late third injection

The duration width and height of the phenomenon were similar to those of the previous groups Here again there is rather close agreement between the start of the temperature change and the second wave of the mydriasis (Table II)

The T_{50} values of the mydriasis and the temperature change again show a larger probably significant difference

The time relations of the degeneration vasoconstriction measured at the tip and the base of the same ear Group VI)

This experiment was intended to show a difference in degeneration time depending on the length of the nerve stump The basic assumption was that the adrenergic nerve terminals in the base and in the tip of the ear are virtually equal and that the duration of their degeneration release would be equal too One cannot expect to



tween denervated and decentralized sides

measure differences between tip and base in starting time of the vasoconstriction because when the central artery at the base of the ear contracts the whole ear becomes cold But if the vasoconstriction starts earlier at the base of the ear it has end earlier than at the tip The base would be warm again while the tip would still be cold This we thought should be possible to see

Two kinds of experiments were made In some the temperature difference between the ears was recorded at the tip as well as at the base (Fig 1) One side was denervated, the other decentralized In other animals the ganglion was removed on both sides and the differences between the room temperature (around 24° C) and the tip and the base of each ear were separately recorded The distance between the thermojunctions at the tip and at the base was 5—7 cm

Since we were interested in the end of the phenomenon, the animals were left in their cages after operation and recording started only 27—30 hrs after denervation Since we had no value for the temperature before the onset of vasoconstriction, we used as zero line the highest temperature that could be recorded after complete elimination of *vasoconstriction by 1—2 mg/kg mecamylamine i m* 45—48 hrs after denervation Later injections did not give higher temperature

It turned out to be impossible to define the end of the vasoconstriction by simple observation because the ear tended to stay cold An attempt was made to eliminate circulating catecholamines by blocking release from the adrenal medullar chromaffin cells by repeated injections of hexamethonium 10 mg/kg

TABLE III The effect of repeated injections of mecamylamine (1—2 mg/kg) on the vasoconstriction towards the end of the degeneration release of transmitter. Base and tip measurements were made on each ear and always showed the same results.
T_1 Time of first injection, hours after operation
E_1 Effect of injection 1
$I_{1,2}$ Interval between first and second injection, hours
E_2 Effect of injection 2
etc.

Symbols — zero or small effect
± about half maximum effect
+ full effect

	Exp no	T_1	E_1	$I_{1,2}$	E_2	$I_{2,3}$	E_3	$I_{3,4}$	E_4
Bilateral ganglionectomy	1	27	—	6	—	6	±	6	+
ear — room temperature	2	28	—	6	—	6	±	6	+
difference	3	30	±	4	+	4	+	8	+
	4	29	—	4	±	4	+	8	+
	5	30	—	5	±	5	±	6	+
Right ganglionectomy	6	29	—	5	±	5	+	8	+
left sympathotomy	7	28	±	5	±	5	+	8	+
right — left ear difference	8	30	—	5	—	5	+	6	+

the effect of the drug was of short duration (0.5—1 hr) and there was considerable tachyphylaxis: the third injection of hexamethonium was already without any influence on the temperature. The same tachyphylaxis was observed also with smaller doses (0.25—0.5 mg/kg). We therefore changed to mecamylamine hydrochloride 1—2 mg/kg which was of longer duration (1.5—2.5 hrs) and showed less tachyphylaxis.

Table III E_3 shows that in 5 of 8 animals mecamylamine caused complete elimination of the vasoconstriction 37—40 hrs after the denervation. The base and the tip temperature always changed synchronously and in the same direction. It never happened that the base returned to zero line without the tip also returning completely. The only difference between the tip and the base was in the height of the phenomenon which was larger at the tip.

Table III shows that the time intervals between the first three injections were on an average about 5 hrs (the last interval is less important because the degeneration vasoconstriction in most cases has come to an end already earlier).

The duration of the effect of each mecamylamine injection was around 2 hrs. Hence, during each interval there were on an average 3 hrs during which the degeneration vasoconstriction at the tip and/or at the base could have ended without one noticing it because locally released transmitter could have been supplanted by circulating catecholamines. In other words any difference in the end points of the phenomenon between the tip and the base of the ear which exceeds 3 hrs would have been noticed. In cases 3 and 4 this time was 2 hrs only.

It might be pointed out that mecamylamine given at 27—30 hrs (and earlier) did

not eliminate vasoconstriction This of course indicates that vasoconstriction at that time was not due to circulating catecholamines

Discussion

Recovery of tone of the ear vessels after denervation has been studied and discussed thoroughly by Grant (1929—31 1931—22 1935—36) who quotes earlier similar observations Grant made no continuous observations in the critical time period covered by us and did not distinguish the transient early vasoconstriction from the later one which he attributes to circulating catecholamines acting on a super sensitive vessel Emmelin and Ohlin (1969) recognized the true nature of the phenomenon That the vasoconstriction studied by them and us is analogous to the degeneration contraction of the nictitating membrane of the cat the periorbital muscle of the rat and the iris dilator of the rabbit is indicated by three findings

1) It is transient

2) It is not counteracted by ganglion blockers presumably affecting the levels of circulating catecholamines and blocking intact nervous pathways to the vessel

3) It is delayed by bretylium like the other phenomena mentioned Thus we feel entitled to call it degeneration vasoconstriction

The time between ganglionectomy and the start of the vasoconstriction in the experiments of Emmelin and Ohlin was about 22 hrs We found a similar figure 20 31 ± 1 26 (S E M) hrs

The degeneration vasoconstriction of the ear vessels was not quite consistent It was not typical in 6 animals out of 26 Possible explanations for such experiments are

1) Feldberg (1926) stresses the fact that the rabbit ear gets its sympathetic inner vation partly from the stellate ganglion Some of these branches bypass the superior cervical ganglion Hence removal of the latter will cause only partial denervation Large anatomical variations do occur

2) The efferent branch to the ear comes from the side of the spindle shaped ganglion and can easily be torn during dissection When torn from the ganglion it might carry ganglion cells along so that the denervation would be only partial also for this reason. There could also be accessory cells outside the ganglion

A phenomenon disturbing the measurements was the spontaneous waves of vaso-constriction with their changing amplitude and length They are due to changes in the denervated ear since they can be seen also in the ear room temperature dif ference Their origin is unclear It seems unlikely that they are due to varying con centration of circulating catecholamines and more likely that they represent a myogenic oscillation of the smooth muscle They have also been seen by Emmelin and Ohlin (1969)

What determines the time lag between removal of the cell body and the de generation release of transmitter at the terminals? Our tip-base experiments indicate that the news of the missing cell body travels the 5—7 cm from base to tip of the

rabbit ear in at most 2—3 hrs maybe even much faster The fastest transport of adrenergic granules observed by Dahlström (1967) is 1 cm/hr in the cat but only 0.2 cm/hr in the rabbit. Livett *et al* (1968) found 0.25 cm/hr in the cat splenic nerve It seems improbable therefore that the travel rate of transmitter granules determines the rate of transmission of the denervation information. the message of the interruption of the axon. Lasek (1968) using the cat dorsal root and Sjöstrand (1969) using the rabbit vagus have found considerably higher rates of axonal transport of protein about 2 cm/hr (Lasek) and 1.7 cm/hr (Sjöstrand) These rates are more compatible with our findings. It should be remembered however, that the 2—3 hrs for travel of information from base to tip is an upper limit and that a much faster transfer is quite compatible with our data On the other hand we cannot exclude the possibility that the terminals at the tip degenerate more easily than those at the base which would allow a longer time for transfer of information from base to tip Our calculations are based on the assumption that the properties of the terminals at the tip and the base do not differ

If the rate of transmission of denervation information is as high as our data seem to indicate the time lag between axotomy and degeneration cannot be accounted for by the time of travel of the information. There must then be at least one other component in the latency Lundberg's (1970b) finding that local injection of bretylium delays the degeneration contraction indicates that one other component could be a process in the periphery One can draw the same conclusion from Emmelin's data (1968) on degeneration secretion where the delay between operation and secretion is far longer than can be accounted for by the length of the stump

According to our results the degeneration vasoconstriction starts about 6 hrs after the degeneration mydriasis In accordance with the discussion above it is improbable that the difference is due mainly to length differences in the degenerating nerve stumps It is more probable that there are real differences between the terminals in the dilatator and those in the artery

It is interesting to compare our data with the results of Emmelin (1968) from the cholinergic auriculotemporal nerve to the parotid gland of the cat His data do not allow a reliable estimate of the rate at which the information travels but are compatible with rates ranging from 4—10 mm/hr The estimates for both our systems are highly uncertain but it is possible that the rate is faster in the adrenergic nerve.

Earlier findings in the rat periorbital muscle and the rabbit pupil indicate (Lundberg 1970a, b Treister and Barany 1970b) that bretylium causes a longer delay of the degeneration release the closer it is given to the expected start of the phenomenon. Probably this is due to gradual loss of bretylium from the nerve ending The loss can be made up by renewed injection and thus delays the transmitter release again. The present results show that the effect of bretylium on the degeneration vasoconstriction was very similar

1) Two injections of bretylium given at the time of denervation and 8 hrs later caused a delay of about 4 hr (group III Table I) Adding a third injection just

before the start of the already delayed phenomenon (around 20 hrs after denervation) caused a further delay of about 5 hrs and the phenomenon started only 29 7 hrs after denervation (group V, Table II)

2) The delaying effect of a single injection of bretylium around 16 hrs after denervation (group IV) was similar to the delaying effect of the two earlier injections in group III

In the groups in which bretylium was injected early during the degeneration mydriasis the start of the temperature change occurred 1—2 hrs after the start of the second wave of the mydriasis This closeness could point to the disappearance of bretylium from the nerve terminals as the factor which starts the delayed phenomenon However, if $T_{50\%}$-values are used, which are less uncertain than the T_0-values the difference is larger and vasoconstriction starts around 3 hrs later than the mydriasis ($P < 0 05$) Hence, an additional factor, maybe differences between the terminals in the two organs cannot be excluded

Our main conclusion is

The latency between axotomy and degeneration release of transmitter in the adrenergic system is not only due to the time taken for 'the news of the missing cell body' to reach the terminals but comprises a time which is different for terminals in different organs

Supported by grants EY 00231 from the National Eye Institute US PHS and grant K69 14X 733 04C from the Swedish Medical Research Council

References

BENMILOUD U and U S v EULER Effects of bretylium reserpine, guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland *Acta physiol scand* 1963 *59* 34—42

DAHLSTROM A and J HAGGENDAL Studies on the transport and lifespan of amine storage granules in the adrenergic neuron system of the rabbit sciatic nerve *Acta physiol scand* 1967 *69* 153—157

EMMELIN N I NORDENFELT and C PEREC Rate of fall in choline acetyltransferase activity in denervated diaphragms as dependent on the length of the degenerating nerve *Experientia* (Basel) 1966 *22* 725—726

EMMELIN N Degeneration secretion from parotid glands after section of the auriculo-temporal ... 418

... as dependent on the length of ... 142—145

... t ear during degeneration of its sympathetic nerve supply *Quart J exp physiol* 1969 *54* 207—210

FELDBERG W The peripheral innervation of the vessels of the external ear of the rabbit *J Physiol* (Lond) 1926 *61* 518—529

GRANT A T Observation on local arterial reactions in the rabbit ear *Heart* 1929—31 *15* 257—280

GRANT R T Further observations on the vessels and nerves of the rabbit ear with special reference to the effects of denervation *Clin Sci* 1935—36 *2* 1—33

GRANT R T E F BLAND and P D CAMP Observation on the vessels and nerves of the ... 3 *16* 69—101

K ... tent of the cat nic ... *J Pharmacol exp*

LANGER S Z The degeneration contraction of the nictitating membrane in the unanesthetized cat *J Pharmacol exp Ther* 1966 *151* 66—72

LASEK R Axoplasmic transport in cat dorsal root ganglion cells as studied with (^{3}H) L leucine *Brain Res* 1968 *7* 360—377

LIVETT B G L B GEFFEN and L AUSTIN, Proximo-distal transport of C^{14} noradrenaline and protein in sympathetic nerves *J Neurochem* 1968 *15* 931—939

LUNDBERG D, Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat *Acta physiol scand* 1969 *75* 415—426

LUNDBERG D Interactions between inhibitors of the axonal amine pump and bretylium in the degeneration release of sympathetic transmitter *Acta physiol scand* 1970a, *78* 503—521

LUNDBERG D Bretylium and the degeneration contraction of the sympathetically innervated periorbital smooth muscle in the rat *Acta physiol scand* 1970b *79* 411—422

MALMFORS T and C SACHS, Direct studies on the disappearance of the transmitter and changes in the uptake storage mechanisms of degenerating adrenergic nerves *Acta physiol scand* 1965 *64* 211—223

SJOSTRAND J., Rapid axoplasmic transport of labelled proteins in the vagus and hypoglossal nerves of the rabbit *Exp Brain Res* 1969 *8* 105—112

SMITH C B U TRENDELENBURG S Z LANGER and T H TSAI The relation of retention of norepinephrine H^3 to the norepinephrine content of the nictitating membrane of the spinal cat during development of denervation supersensitivity *J Pharmacol exp Ther* 1965 *151* 87—94

TREISTER G and E H BARANY, Mydriasis and intraocular pressure decrease after cervical ganglionectomy in the conscious rabbit *Invest Ophthal* 1970a *9* 331—342

TREISTER G and E H BARANY The effect of bretylium on the degeneration mydriasis and intraocular pressure decrease in the conscious rabbit after unilateral cervical ganglionectomy *Invest Ophthal* 1970b *9* 343—355

VAN ORDEN L S III K G BENSCH S Z LANGER and U TRENDELENBURG Histochemical and fine structural aspects of the onset of denervation supersensitivity in the nictitating membrane of the spinal cat *J Pharmacol exp Ther* 1967 *157* 274—283

ZAK S J R H GOOD and R A GOOD A technique for one stage bilateral adrenalectomy in the rabbit *Nature* (Lond) 1957 *179* 100—102

Acta physiol. scand. 1970. 80. 93—106

From the Department of Physiology, University of Goteborg Sweden

Background of Increased Flow Resistance and Vascular Reactivity in Spontaneously Hypertensive Rats

By

Bjorn Folkow, Margareta Hallbäck, Yen Lundgren and Lilian Weiss

Received 3 February 1970

Abstract

Folkow, B, M Hallbäck, Y Lundgren and L Weiss *Background of increased flow resistance and vascular reactivity in spontaneously hypertensive rats* Acta physiol scand 1970 *80* 93—106

The hindquarters of a spontaneously hypertensive rat (SHR and a matched normotensive control rat (NCR) were perfused at a constant rate of flow with oxygenated plasma substitute in 15 paired experiments As is the case in the entire systemic vascular bed (Folkow *et al* 1969), flow resistance was raised even during maximal dilatation in SHR ($p < 0.001$) almost in proportion to their raised blood pressure Graded noradrenaline (NA) infusions showed identical NA "thresholds while SHR displayed a steeper curve relating log NA dose to resistance response ($p < 0.001$) and a greater maximal contractile strength of the resistance vessels ($p < 0.001$) These characteristics of the resistance curves for SHR and NCR were compared with those mathematically deduced for two hypothetical vessels identical except for the presence in one of them of a 30 per cent increase of its media thickness encroaching upon its lumen even at complete relaxation In all essential points the relationships between the two sets of curves were the same while no other type of vascular change structural or functional could alone reproduce all the characteristics of the SHR curve The results therefore suggest the presence of an increased contractile wall mass in the systemic resistance vessels of SHR encroaching upon their lumina even during maximal dilatation. Without necessitating any increased smooth muscle activity the haemodynamic effects of the proposed structural change can largely alone account for the raised resistance and increased vascular "reactivity in SHR.

Studies of regional vascular beds in man suggest that the increased systemic resistance in essential hypertension may predominantly be due to a structural change of the resistance vessels (Folkow 1956 Folkow Grimby and Thulesius 1958 Conway 1963) Thus even during complete vascular relaxation forearm resistance to flow was raised almost to the same extent as the resting blood pressure It was further illustrated theoretically how the presence of an increase of wall mass in relation to the lumen thus enhancing the wall/lumen ratio of the resistance vessels will increase their "reactivity to constrictor influences without necessitating any enhanced sensitivity or tension development of individual contractile elements (Folkow 1956) The reason is that any increase (decrease) of this ratio leads to exaggerated (reduced) luminal narrowings for given degrees of smooth muscle shortening

In some of the experiments tests were made to investigate whether the responses of the two vascular beds differed when low concentrations of either angiotensin or vasopressin were given. Moreover after completion of the series of NA infusions supramaximal amounts of vasopressin

ponses produced

NA

the tissue mass

it Considerable oedema occurred only if very intense pressor responses were maintained for long periods and there was no difference between the two animals in this respect

The levels of minimal flow resistance and the vasoconstrictor responses to NA in the two hindquarter preparations were then calculated and plotted with the log NA dose per ml perfusate on the abscissa and the flow resistance as PRU_{100} on the ordinate after subtraction of the small resistance offered by the aortic cannulas between the point of pressure measurement

The *maximal response* was defined as the highest pressure level reached during constant flow perfusion implying that the high distending pressure then curtailed further active shortening even though supramaximal concentrations of vasoconstrictor agents were present In contrast to the maximal constriction obtained at the more physiological type of constant pressure perfusion which more resembles an isotonic contraction and allows for an unhindered shortening the constant flow principle allows for a fair comparison of the strength of contraction of the resistance vessels, as determined by their smooth muscles and as influenced by the current wall/lumen ratio For such reasons resistance is represented by the perfusion pressure in the diagrams shown below

For each pair of rats the percentage differences in resting blood pressures, in flow resistance at maximal dilatation and at comparable levels of NA concentrations were deduced Also the maximal pressor responses and 50 per cent of these maximal responses (M_{50}) were deduced Mean values for the two groups of animals and for the percentage differences between them were calculated and statistically analyzed

Results

The resting blood pressure of SHR and NCR, as measured in the right carotid artery was 217 ± 6 mm HG (mean $\pm$ S E) and 153 ± 6 mm Hg respectively With allowance for the fact that pressure measurement at this site yields values that are some 25—35 mm Hg higher than normal (see Methods) these pressure values correspond well to those measured in the same strains of animals by femoral artery cannulation in a recent series of experiments (Folkow *et al* 1969 1970)

During complete vascular relaxation mean flow resistance in the hindquarter preparations was $3\ 2 \pm 0\ 2$ PRU_{100} in the SHR and $2\ 4 \pm 0\ 2$ PRU_{100} in the NCR. This implies a structurally determined difference in resistance of about 35 per cent ($p < 0\ 001$) which is almost as large as the difference in resting blood pressure between the two groups of animals (see also Table I)

With respect to threshold sensitivity to NA defined as a 25 % rise in resistance from the state of maximal dilatation there was no significant difference between SHR and NCR (Table I) if anything there was a tendency for NCR to respond at slightly lower NA concentrations In the smaller group of animals where threshold sensitivity to angiotensin and vasopressin was compared as well no difference between hypertensive and normotensive animals was ever observed In other words there was no evidence of any increased smooth muscle sensitivity of the SHR resistance vessels to vasoconstrictor agents

TABLE 1 [illegible]

sponse (M_{50})

	Arterial blood pressure mm Hg	PRU_{100} at max dil	Threshold µg NA/ml	Corrected slopes of curves as tangent of the angle	Maximal response mm Hg	NA dose for M_{50} µg/ml
NCR	153±6	2 4±0 2	0 08±0 01	2 55±0 3	201±8	0 37±0 05
SHR	217±6	3 2±0 2	0 09±0 01	4 0±0 4	287±9	0 30±0 05
% increase in SHR	44 %±6	35 %±5	7 %±12	56 %±10	45 %±7	−18 %±6
Significance	p<0 001	p<0 001	0 5>p>0 4	p<0 001	p<0 001	p<0 05

Once, however, the dose of the vasoconstrictor agent was further increased and the resistance response grew stronger, so that the presence of an increased wall/lumen ratio could appreciably accentuate the extent of luminal reduction a clear difference between NCR and SHR became evident A typical individual experiment is shown in Fig 1 The general principles are, however, best illustrated by the two curves in the left part of Fig 2, which are based on the average resistance responses of all the pairs of animals to a series of increasing NA concentrations After plotting of each pair of experimental curves the percentage difference between SHR and NCR with respect to resistances at maximal dilatation was deduced as well as the NA dose

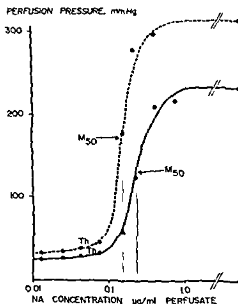
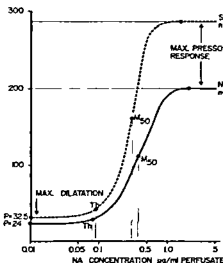


Fig 1 Constant flow perfusion of the parallel coupled hindquarters from one SHR and one NCR showing the effects on the perfusion pressure of noradrenaline infusion at increasing concentrations, from subthreshold to supra maximal levels

The points representing individual measurements are used for the construction of the "resistance curves" where the solid line represents NCR and the dashed line SHR Th denotes "threshold" i e a 25 per cent increase of flow resistance from the state of maximal dilatation Mso denotes 50 per cent of the maximal pressor (resistance) response For details see text



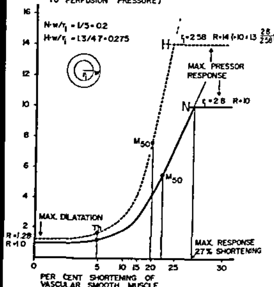


Fig 2 The left part shows the average "resistance curves" for SHR and NCR (see Fig 1) based on the results of 15 paired experiments

The right part shows the mathematically deduced "resistance curves" for two hypothetical resistance vessels, H and N, where H differs from N only in the respect that its media thickness is supposed to be increased 30 per cent, encroaching upon its lumen even at maximal relaxation

Note the striking similarities between the relationships of the two sets of "resistance curves" with respect to 1 Resistance at maximal relaxation, 2 "Threshold" (Th), 3 Steepness of curves 4 50 per cent of the maximal pressor (resistance) response (M_{50}) and 5 Maximal pressor (resistance) response For details see text

producing 25 % increase of the resistance In the same way the percentage differences were deduced for the maximal response and half of the maximal response, M_{50}, as well as for the slopes of the steep parts of the curves (the tangent of the angle) It should in this connection be stressed that M_{50} is not identical with E_{D50} in conventional dose-response curves for *e g* strips of smooth muscle The reason is that the increasing wall/lumen ratio of the constricting vessels in an accelerated way potentiates the luminal reduction for a given degree of smooth muscle shortening Therefore the true E_{D50} for the contracting smooth muscles must be placed somewhere below M_{50} on the ordinate

Moreover, for the calculation of the tangent of the angle which describes the curve steepness, correction was made for the fact that the SHR curve starts from a higher resistance Other things being equal a higher initial resistance gives a proportionally steeper angle in a coordinate system of the type used in Fig 1 and 2 The tangent of the angle for SHR given in Table I is however corrected for this "error" and the difference between them is nevertheless pronounced (see below)

The mean figures for the five values mentioned above were calculated for the NCR and the SHR and from these key figures the simplified curves in Fig. 2 were constructed (see also Table I). Besides the difference in resistance at maximal dilatation ($p < 0.001$) and the almost identical NA thresholds a comparison of these dose response curves reveals a 56 per cent increase of steepness in SHR ($p < 0.001$). In addition these animals also display a larger maximal response ($p < 0.001$), which is increased largely in proportion to their higher level of 'resting' blood pressure (see also Table I). Lastly, M_{50} for SHR is somewhat displaced to the *left* of that for NCR ($p < 0.05$) despite the fact that their thresholds to NA are almost identical, if anything slightly higher for SHR.

The presence of a higher flow resistance even during complete vascular relaxation, combined with an unchanged sensitivity to NA but with a steeper resistance increase and a higher maximal pressor response to large NA concentrations strongly suggests the presence of a structural increase of contractile wall mass as related to the lumen in the resistance vessels of the hypertensive animals. The increased wall thickness is evidently of such a nature that it encroaches upon the lumen even during maximal vasodilatation.

However the functional impact of such a structural change is best illustrated by exact computations of resistance changes in idealized 'model' vessels with arbitrary but reasonable values for vascular design at the state of complete relaxation as shown by *the curves in the right part of Fig. 2. The following assumptions and calculations* have here been used.

1. The ratio between wall thickness (w) and internal radius (r) of a 'normal' resistance vessel (N) is set at 1 : 5 at maximal dilatation. This ratio is in agreement with Van Citters' values for normally distended and completely relaxed arterioles (Van Citters 1966).

2. This same ratio of a 'hypertensive' resistance vessel (H) is assumed to be 1.3 : 4.7 because of a structural increase of contractile wall mass amounting to a 30 per cent thickening of the wall. This wall thickening has by encroaching upon the lumen reduced r_i from 5 to 4.7, *i.e.* by 6 per cent. It follows from Poiseuille's law that the flow resistance of H is 1.28 times higher than that of N $(5/4.7)^4$ at complete vascular relaxation.

3. The wall mass is considered to be a constant when N and H constrict, which seems reasonable and which is supported by direct observations of normal vessels (Baez 1969). It is further assumed that constriction is initiated from the outermost muscle sheath which certainly is the case with normal vasoconstrictor fibre control since these fibres make contact only with the adventitial muscle surface. This assumption seems justified also when the resistance vessels are exposed to exogenous NA for the following reason. A recent study of the portal veins of the rat (Johansson *et al.* 1970) suggests that most of the α receptors important for the response to exogenous NA are located to the directly innervated effector cells. The smooth muscles of this vessel like those of the resistance vessels belong to the 'single unit' type and also in other respects they exhibit many similarities …

For the resistance vessels this would imply that the constriction caused by exogenous NA pushes inner tissue layers towards the lumen which will in an exaggerated way reduce the lumen the more pronounced the smooth muscle shortening becomes and also the bigger the wall mass is to start with

4 The extent of shortening of the outermost muscle sheath in these hypothetical resistance vessels is plotted along the abscissa as per cent reduction in length and the calculated increases of flow resistance for both N and H are plotted along the ordinate The figures for flow resistance are computed in the following way For each reduction of the outer circumference (*i e* of smooth muscle length) the total vascular transverse section area is calculated from the reduced external radius From this area the constant surface area of the wall is subtracted the remainder being the transverse section area of the lumen This allows a calculation of the internal radius r and of the flow resistance for each level of smooth muscle shortening

5 As in the experiments it is assumed that N and H are perfused at constant flow which means that their constriction enhances the pressure load on their walls until the limit set by their maximal contractile strength is reached For N this limit is arbitrarily assumed to be reached when resistance (and pressure) has increased from 1 to 10 This approximately corresponds to the situation in NCR As the 30 per cent increase of wall thickness in H was assumed to mainly concern the contractile elements H and N being equal in all other respects such as sensitivity and contractile strength per unit contractile element H will be able to reach a 30 per cent higher level of maximal pressor response for this reason alone Further if Laplace s law is considered and the inner radius is assumed to be representative for the deduction of wall tension the effect of the differences in r_i between H and N must also be considered in calculations of the maximal pressor response of H At the actual levels of *maximal resistances r can be calculated to be about 2 58 in H and 2 80 in N their* ratio being about 0 92 It follows that the maximal pressor response of H is reached at a resistance of $\frac{10 \times 1\ 3}{0\ 92} = 14$ However the inner non contractile wall layers are probably unloaded by the contracting muscles so that they hardly contribute to wall tension in this situation If so the true radius for applying the Laplace law would be larger than r for both vessels and the ratio between them would then be correspondingly closer to 1 The theoretical maximal pressor response of H would then be slightly smaller than 14

6 For both H and N 50 per cent of the maximal pressor resistance) response M_{50} was calculated It should here be stressed that the difference in wall/lumen ratio determines the different steepness of the resistance curves while mainly the different amounts of contractile wall mass determines the differences in the maximal pressor response From this it follows mathematically that M_{50} for H falls to the left of that for N, despite identical thresholds and degrees of smooth muscle shortening along the abscissa

7 The abscissa for the curves illustrating the experimental results is given as l g NA dose For the theoretical vessels on the other hand the abscissa is given as the

percentage decrease of smooth muscle length However log NA dose and decrease of smooth muscle length are normally related to each other in the form of the S formed dose response curve characterizing e g a contracting smooth muscle strip Therefore to make the scales of the two abscissae as equal as possible in the two parts of Fig 2 a typical log NA dose response curve for vascular smooth muscle (aortic strip) was utilized for transformation The response on the ordinate was then divided in a linear fashion in per cent of the maximum and each of these points was transferred *via* its section with the S formed curve to the abscissa By this procedure the proportions between *e g* consecutive 5 per cent shortenings of the contractile elements of H and N could be made more directly comparable to the actual shortenings of the vascular smooth muscles that were caused by the different NA doses in the experiments This explains the particular scale for smooth muscle shortening used as the abscissa for the right hand part of Fig 2

From these theoretical considerations the resistance curves for the hypothetical resistance vessels H and N were deduced as shown in the right hand part of Fig 2 It is seen from Fig 2 that the relationship between on the one hand the experimental curves (left) and the mathematically deduced curves (right) on the other are strikingly similar in their five key points

1 There is a difference in flow resistances even at complete vascular relaxation those of H and SHR being both higher

2 The NA thresholds are identical

3 There is a difference in curve steepness the curves of H and SHR being steeper

4 There is a difference in maximal pressor response both H and SHR exhibiting stronger responses

5 M_{50} for both H and SHR is displaced to the left of that for N and NCR despite equal thresholds

In fact even quantitatively the relationship between the two sets of curves is so closely similar that it is tempting to assume that even the magnitude of the causative factor may be roughly the same in H and SHR *i e* corresponding to perhaps a 30—40 per cent increase of media thickness in the SHR resistance vessels encroaching upon their lumina even at complete relaxation

Discussion

In the present experiments a comparison of the flow resistance in the hindquarters of spontaneously hypertensive rats (SHR) and matched normotensive controls (NCR) was performed from the level of maximal dilatation to that of maximal vasoconstriction. The results are in complete agreement with the view that the increased vascular reactivity and flow resistance in well established hypertension during rest may be entirely caused by a structural increase of wall thickness in the systemic resistance vessels encroaching upon their lumina even at maximal dilatation (Folkow 1956)

Such a view is supported by the following experimental observations concerning the haemodynamic characteristics of the resistance vessels When compared with NCR SHR exhibited 1) A raised flow resistance (about 35 per cent) also at maximal dilatation ($p<0.001$)

2) An unchanged sensitivity to threshold amounts of vasoconstrictor agents ($0.5> p>0.4$)

3) Exaggerated resistance responses to suprathreshold amounts of such agents expressed as an increased steepness (56 per cent) of the resistance curve ($p<0.001$)

4) An increased strength of contraction expressed as a raised maximal pressor response (about 40 per cent) to supramaximal amounts of vasoconstrictor agents during constant flow conditions ($p<0.001$)

5) A shift towards the left of the resistance response that corresponded to 50 per cent of the maximum ($p<0.05$)

The implications of such a series of haemodynamic characteristics of the SHR resistance vessels becomes particularly clear by comparing them to mathematically deduced resistance changes in two hypothetical model vessels", setting out from arbitrary but biologically reasonable values for the structurally determined wall/lumen ratio It was here assumed that a hypothetical "hypertensive" vessel H differed from a normotensive one, N only in the respect that H displayed a 30 per cent increase of media thickness encroaching upon its lumen even at complete wall relaxation The deduced resistance curves for these model vessels exhibited precisely the five differences and similarities mentioned above that characterized the experimental findings concerning the SHR and NCR resistance vessels In fact the relationship between the H—N resistance curves and that between the SHR—NCR resistance curves was even quantitatively so close as to suggest that the extent of structural change of the SHR resistance vessels may have been of a similar order of magnitude as that assumed to be present in H

The question arises however whether any other type of vascular change, structural or functional may explain all the mentioned characteristics of the relationship between the SHR and NCR resistance vessels A thickening of only *non*-contractile wall elements or a mere water logging of the wall (Tobian and Binion 1952) could explain most differences but *not* the considerable increase of maximal contractile strength of the SHR resistance vessels beyond the minor gain that according to Laplace's law may be inherent in the smaller internal radius of SHR (see Results and right hand part of Fig 2

A hypothetical rarification of the resistance vessels or a reduction of their size at an unchanged wall lumen ratio could explain the increased resistance at maximal dilatation and the unchanged NA threshold but not the other haemodynamic characteristics of the SHR vessels

An increased smooth muscle sensitivity to vasoconstrictor agents could not explain any of the characteristics of the SHR vessels Again an increased ability per square unit contractile tissue to develop active tension in a vessel of unchanged design would neither explain the increased resistance at maximal relaxation nor the displacement

of M_{50} towards the left The reason is that in such a hypothetical case both the curve steepness *and* the maximal pressor response would be proportionally increased then M_{50} would not be displaced

In other words it appears as if only the presence of an increased wall thickness which mainly involves the smooth muscle coat and encroaches upon the lumen of the resistance vessels even at maximal dilatation can *alone* explain all the haemodynamic characteristics of the SHR resistance vessels It should be stressed that many morphological studies (see *e g* Pickering 1968) have described hypertrophic changes of the arterial arteriolar walls in well-established hypertension *i e* an increase of wall thickness when related to the lumen However such studies alone can hardly reveal whether the wall encroaches upon the lumen al o at maximal vascular relaxation or whether other types of haemodynamically important consequences are involved several alternative types of morphological change are *a priori* possible in this respect (Folkow Grimby and Thulesius 1958) and only haemodynamic studies can settle which alternative that is relevant In the forearm and hand of subjects with essential hypertension it has been shown that flow resistance is increased even at maximal relaxation of the vessels (Folkow 1956 Folkow Grimby and Thulesius 1958 Conway 1963 Sivertsson and Olander 1968) Moreover results on the hindlimb vessels of cats (Folkow and Sivertsson 1968) and on the hand vessels of man (Sivertsson and Olander 1968) indicate first that arterial vessels within few weeks change their wall thickness when the load is changed and second that the steepness of the resistance curve is changed accordingly while the threshold to noradrenaline remain unchanged

These studies of regional vascular beds have recently been extended to include the entire systemic vascular bed of SHR as compared with that of NCR. It was shown that even at complete vascular relaxation the systemic flow resistance is significantly higher in SHR ($p<0.001$) and largely in proportion to the raised pressure level (Folkow *et al* 1969 1970)

Studies on the isolated perfused mesenteric arterial tree from the New Zealand strain of hypertensive rats show that these Windkessel vessels exhibit increased responses to noradrenaline (NA) injection but it was not determined whether this was due to *e g* an increased wall/lumen ratio or to an increased NA sensitivity McGregor and Smirk 1968 In the same preparation of larger arteries from the Okamoto strain of SHR the entire dose-response curves were de ermined Haeusler and Haefely 1970 and these results were taken to indicate that an increased smooth muscle sensitivity was present On the other hand the differences between at least some of the dose-response curves of the hypertensive and normotensive animals in these latter experiments on larger arteries may suggest that they too were entirely or to a major extent consequences of an increased wall/lumen ratio

While McGregor and Smirk (1968) report that the flow resistance of the large arteries from the New Zealand type of hypertensive rats was raised even at complete dilatation this was apparently not the case with those from the Okamoto strain of SHR studied by Haeusler and Haefely (1970 The true resistan e vessels of the

Okamoto SHR display, as mentioned, a clearly raised resistance to flow even at complete smooth muscle relaxation with respect to both the entire systemic circuit and the hindquarter vessels. It should in this context be stressed that despite some overlap the resistance vessels and the "Windkessel" vessels represent two different sections of the vascular bed, both structurally and functionally (see e.g. Mellander and Johansson 1968), and it may be dangerous to draw too close parallels between them with respect to both haemodynamics and smooth muscle characteristics. Luminal reduction is not the primary function of Windkessel vessels and in normal animals they usually become distended and widened when a pressor response is elicited by a constriction of the true resistance vessels. Apart from such differences the large arteries in the study of Haeusler and Haefely (1970) displayed a clearly raised maximal strength of contraction when exposed to supramaximal NA doses during constant flow perfusion and the dose response curve was steeper. These findings indicate that also the arterial Windkessel vessels may display a wall thickening involving their smooth muscle coat, though not necessarily encroaching upon their lumina as is the case with the resistance vessels.

To summarize, the present results suggest the presence of a characteristic and haemodynamically important structural rebuild of the resistance vessels in SHR (see Fig. 2). It appears to be extensive enough as to alone explain both the increased vascular "reactivity" and the raised flow resistance in the resting equilibrium of these animals without necessitating any increase of vascular smooth muscle activity. It is however hardly likely that such a change of vascular design would constitute the in time primary cause of the hypertensive state, though it appears to develop within few weeks after a primary change in pressure load, as mentioned above. This implies such a close association in time between the structural rebuild of the resistance vessels and the hypothetical "trigger" mechanism as to make them almost inseparable. The trigger mechanism may well be a functional, perhaps even intermittent disturbance of neuro-hormonal origin, as initiated e.g. from cortico-hypothalamic levels. It has been shown that intermittent weak stimulations of the hypothalamic defence area in rats gradually lead to a hypertensive state that is present also upon withdrawal of all stimulation (Folkow and Rubinstein 1966).

The question then arises what might be the genetically linked factor responsible of the high blood pressure in SHR and of "primary" (essential) hypertension in man. It is possible that the genetic factor is constituted by some type of increased neuro-hormonal activity, acting as an abnormally powerful trigger mechanism which causes structural changes in vessels responding to a normal extent in this respect.

There is however also another possibility of considerable interest, which might be relevant for any or both of the mentioned types of "primary" hypertension in rats and in man. It is known that the cardiovascular system also of normotensive subjects is frequently exposed to intermittent loads of considerable power during routine daily life, in the form of emotionally elicited cortico-hypothalamic discharge patterns (e.g. Folkow 1961, Hinman, Engel and Bickford 1962, Charvat, Dell and Folkow 1964, Bevan, Honour and Stott 1969). This can certainly also be the case in animals at

least in certain environmental situations as has been shown in mice (Henry, Meehan and Stephens 1967)

It is therefore not impossible that the genetic factor responsible of the high blood pressure in SHR as well as of essential hypertension in man might be constituted by a moderately increased tendency of tissues to respond with adaptive 'hypertrophic' changes If this is the case, also the blood vessels would be expected to do so even in response to intermittent functional loads that may in extent be fully normal A gradual development of a structurally based hypertensive state would then be expected whenever the *per se* normal 'trigger would tend to become intensified and/or the genetic element especially strong (see also Folkow 1956 Folkow, Grimby and Thulesius 1958) It should in this context be recalled that subjects with essential hypertension seem to be characterized by a prevalence for mesomorphical body build (Robinson and Brucer 1940)

However, whether the structural rebuild of the resi tance vessels really constitutes the genetically linked element or not or whether it is strictly primary" or "second ary in nature its early involvement and importance for the creation and further development of a hypertensive state seems obvious From the point of view of the individual blood vessel, this type of "adaptive increase of wall/lumen ratio seems as appropriate and normal as the hypertrophy of skeletal muscles exposed to increased load For the cardiovascular system as a whole however it has unfortunate consequences since its apparent generalization to all or most resistance vessels (Folkow *et al* 1969 1970) seems to constitute the very basis for the maintenance and further development of a hypertensive state which by inviting secondary pathological derangement, makes the situation potentially serious

We are most indebted to the Genetics Units National Institute of Health Bethesda USA, for

05675 08) and from the Swedish Medical Research Council B70-14X 16-06B) Ab Hassle Goteborg Sweden has generously covered part of the expenses for technical assistance

References

BAEZ S Simultaneous measurements of radii and wall thickness of microvessels in the anesthetized rat *Circulat Res* 1969 *25* 315—329

BEVAN A T A J HONOUR and F H STOTT Direct arterial pressure recording in unrestricted man *Clin Sci* 1969 *36* 329—344

CHARVAT J P DELL and B FOLKOW Mental factors and cardiovascular diseases *Cardiologia* Basel 1964 *44* 124—141

CONWAY J A vascular abnormality in hypertension a study of blood flow in the forearm *Circulation* 1963 *27* 520—529

FOLKOW B Structural myogenic humoral and nervous factors controlling peripheral resist ance *Hypertensive Drugs* Pergamon Press 1956 163—174

FOLKOW B Haemodynamic responses to cortical and hypothalamic stimulation In *The joint symposium on the pathogenesis of essential hypertension (WHO Czechosl Cardiol Soc)* State Medical Publ House Prague 1961 247—255

FOLKOW B, G GRIMBY and O THULESIUS Adaptive structural changes of the vascular walls in hypertension and their relation to the control of the peripheral resistance *Acta physiol scand* 1958 *44* 255—272

FOLKOW B M HALLBACK Y LUNDGREN and L WEISS Structurally based increase of flow resistance in spontaneously hypertensive rats *Acta physiol scand* 1969 Suppl 330 94 Preliminary report)

FOLKOW B, M HALLBÄCK Y LUNDGREN and L WEISS Structurally based increase of flow resistance in spontaneously hypertensive rats *Acta physiol scand* 1970 *79* 373—378

FOLKOW B and E H RUBINSTEIN Cardiovascular effects of acute and chronic stimulations of the hypothalamic defence area in the rat *Acta physiol scand* 1966 *68* 48—57

FOLKOW B and R SIVERTSSON Aspects of the difference in vascular "reactivity" between cutaneous resistance vessels and A—V anastomoses *Angiologica* 1964 *1* 338—345

FOLKOW, B and R SIVERTSSON Adaptive changes in "reactivity" and wall/lumen ratio in cat blood vessels exposed to prolonged transmural pressure difference *Life Sci* 1968 *7* part I 1283—1289

HAEUSLER G and W HAEFELY Pre and postjunctional supersensitivity of the mesenteric artery preparation from normotensive and hypertensive rats *Naunyn Schmiedeberg's Arch exp Path Pharmak* 1970 *266* 18—23

HENRY J P J P MEEHAN and P M STEPHENS The use of psychosocial stimuli to induce prolonged hypertension in mice *Psychosom Med* 1967 *29* 408—432

HINMAN A T B T ENGEL and A F BICKFORD Portable blood pressure recorder, accuracy and preliminary use in evaluating intradaily variations in pressure *Amer Heart J* 1962 *63* 663—668

JOHANSSON B B LJUNG T MALMFORS and L OLSON Prejunctional supersensitivity in the rat portal vein as related to its pattern of innervation *Acta physiol scand* 1970 Suppl 349

MCGREGOR D D and F H SMIRK Vascular responses in mesenteric arteries from genetic and renal hypertension *Amer J Physiol* 1968 *214* 1429—1433

MELLANDER S and B JOHANSSON Control of resistance exchange and capacitance functions in the peripheral circulation *Pharmacol Rev* 1968 *20* 117—196

OKAMOTO K and K AOKI Development of a strain of spontaneously hypertensive rats *Jap Circulat J* (En 1963 *27* 282—293

PICKERING G *High blood pressure* Ed 2 J & A Churchill Ltd London 1968

ROBINSON S C and M BRUCER Body build and hypertension *Arch intern Med* 1940 *66* 393—417

SIVERTSSON R and R OLANDER Aspects of the nature of the increased vascular resistance and increased reactivity to noradrenaline in hypertensive subjects *Life Sci* 1968 *7* part I 1291—1297

TOBIAN L J and J T BINION Tissue cations and water in arterial hypertension *Circulation* 1952 *5* 754—758

VAN CITTERS R L Occlusion of lumina in small arterioles during vasoconstriction *Circulat Res* 1966 *18* 199—204

Acta physiol. scand. 1970. 80. 107—116

From the Department of Pharmacology, University of Uppsala, Sweden

The Degeneration Contraction of a Sympathetically Innervated Smooth Muscle in the Rat after Reserpine, Inhibition of Monoamine Oxidase or Tyrosine Hydroxylase

By

DAG LUNDBERG

Received 5 February 1970

Abstract

LUNDBERG, D *The degeneration contraction of a sympathetically innervated smooth muscle in the rat after reserpine, inhibition of monoamine oxidase or tyrosine hydroxylase* Acta physiol scand 1970 *80* 107—116

Th [illegible] ous rats was measured [illegible] nhibitors reserpine or [illegible] (100 or 200 mg/kg [illegible] argyline (100 or 200 mg/kg) given at 10 hrs after denervation significantly increased the size of the degeneration contraction 200 mg/kg of pargyline tended to delay the onset of the contraction. However pheniprazine, nialamide or pargyline at 100 mg/kg started it prematurely An injection of reserpine (1 mg/kg) given at 2—3 hrs before the expected start of the degeneration transmitter release did not induce any excitatory effect and abolished the degeneration contraction Inhibition of the synthesis of noradrenaline by the methylester of l-α methyltyrosine (H 22/07) given at the time of denervation or 10—12 hrs later did not influence the size or the time course of the degeneration contraction It is concluded that — The degeneration transmitter release is normally influenced by functional MAO Since the potent MAO inhibitors studied did not consistently delay the start of the degeneration contraction it does not seem reasonable that bretylium, which earlier has been found both to possess MAO-inhibiting and delaying effect induces a delay only by inhibiting intraaxonal MAO — There is no significant contribution of newly formed noradrenaline to the degeneration contraction

It is known that during the first few days after division of the postganglionic autonomic nerves there is a spontaneous and transient stimulation of the effector organ which is most probably due to release of stored transmitter from the degenerating nerve terminals (Emmelin and Strömblad 1957 Sears and Barany 1960) The neuron blocking quaternary amines bretylium and beta-TM 10 have been shown to interfere with the degeneration processes in the adrenergic nerves *viz* to delay the start of the postdenervation disappearance of the transmitter or the postdenervation excitatory effect (*bretylium* Benmiloud and Euler 1963 Malmfors and Sachs 1965 Lundberg 1969 Triester and Barany 1970 *Beta TM 10* Langer 1966

Pluchino van Orden III Draskoczy, Langer and Trendelenburg 1970) The bretylium induced delay is probably not caused by a neuron block (Lundberg 1969 and 1970) and the mechanism of the delaying action is still unknown Bretylium and beta TM 10 also possess monoamine oxidase (MAO) inhibiting activity (*bretylium* Mc Coubrey 1962 Kuntzman and Jacobsen 1963, Furchgott and Sanchez Garcia 1966 Giachetti and Shore 1967 Clarke and Leach 1968, *Beta TM 10*· Pluchino et al 1970 Recently Henneman and Trendelenburg (1970) have found that pargyline an inhibitor of MAO delayed the decline of endogenous noradrenaline from the denervated rat submaxillary gland to the same extent as beta TM 10 The authors ascribed the effect to MAO inhibition

In view of the suggested relation between inhibited MAO and delay of the degeneration transmitter release it seemed worthwhile to study the effects of several potent MAO inhibitors on a degeneration contraction of periorbital smooth muscle in the rat earlier described (Lundberg 1969) Phenipiazine, nialamide and pargyline which belong to two different groups of MAO inhibitors were used Each drug started the degeneration contraction prematurely instead of delaying it Furthermore they increased the size of the contraction

Functional MAO thus reduces the degeneration contraction but cannot prevent it It therefore was of interest to test whether in this effector system release of transmitter by reserpine has a visible excitatory effect This was not the case

Another question of interest concerning the degeneration contraction is whether there is any appreciable new synthesis of transmitter in the degenerating nerve endings In order to get some information about this rats were given the methylester of l α methyltyrosine (H 22/07) which inhibits the synthesis of noradrenaline in vivo at the rate limiting step Spector Sjoerdsma and Udenfriend 1965 Corrodi and Hanson 1966 H 22/07 injected during the postdenervation time interval did not significantly influence the time course or the size of the degeneration contraction

Materials and methods

Experimental animal

Male Sprague Dawley rats were used They were kept in normal daylight conditions at around 23° C Commercial rat food pellets no 210 Anticimex Sollentuna Sweden and tap water were provided ad lib

Surgical procedure

Under ether anesthesia the right superior cervical ganglion was extirpated (denervation) On the left side the preganglionic sympathetic trunk was cut decentralization)

Drugs

The following drugs were kindly donated by their manufacturers Nialamide (Niamid®) Pfizer & Co Inc, New York Pargyline hydrochloride MO 911 Eutonyl®) Abbott Lab North Chicago Phenipiazine (Catran® Draco Ltd Lund Sweden l α methyltyrosine methylester hydrochloride monohydrate H 22/07 Hässle Ltd Gothenburg Sweden Reserpine (Serpasil® ampouls with 2 5 mg/ml Ciba Ltd Basel Switzerland

The drugs except reserpine were dissolved in 0 9 NaCl immediately before use The doses refer to the salts

Measurement procedure

The sizes of the palpebral apertures were measured intermittently in conscious animals. The method of measurement and calculations has been described in detail earlier (Lundberg 1969). The curve of the degeneration contraction was constructed by plotting the difference in palpebral aperture between the denervated and the decentralized (control) side at every measurement occasion against the time after denervation.

The degeneration contraction was characterized from the following points of view.

The time course. The times corresponding to 30, 50 and 70 per cent of maximum effect on the ascending part (T_{30a}, T_{50a} and T_{70a}) and those corresponding to 50 and 70 per cent (T_{50d} and T_{70d}) on the descending part of the curve were calculated. T_{30a} and T_{50d} were used as time of start and time of end of the degeneration contraction, respectively.

The slopes of the curve limbs. The differences between T_{70a} and T_{30a} (rise time) and T_{50d} and T_{70d} (fall time) are inverse expressions for the slopes of the ascending and the descending limbs respectively. The asymmetry in the rise time and fall time definition is due to that the contractions usually were not followed long enough to obtain T_{30d} values.

The duration (the width). This is the difference between T_{50d} and T_{30a}.

The height. This is the maximal value noted on the denervated side during the degeneration contraction, no regard being taken to the decentralized (control) side.

The size. Besides the height and the duration, the area covered by the difference curve is an expression for the size of the degeneration contraction. In the experiments with the MAO inhibitors, as distinguished from earlier experiments, the degeneration contraction was supra [illegible]

lowed for equal time intervals after the starts of the contraction.

Student's t test was used for the analysis of significance.

Results

Effects of pargyline, nialamide or phenipraz ine on the degeneration contraction

The results are shown in Table I and Table II. Two series of experiments were performed 1 1/2 years apart. Control rats run simultaneously were given 0.9 percent NaCl instead of the drugs. In the first series 200 mg/kg of pargyline or nialamide was injected s.c. to rats in groups of 5. The drugs were given at the time of denervation (pargyline and nialamide) or at 10 hrs after denervation (pargyline). In each pargyline treated group 2 rats out of 5 died within 15 hrs after the treatment. However, the other animals of these groups and the rats of the nialamide group were in good condition throughout the experiment. In the second series groups of rats (n = 5) were given pargyline or nialamide, both drugs at 100 mg/kg, or 10 mg/kg of pheniprazine. The drugs were injected s.c. at the end of the operation or, as in one pargyline group, at 10 hrs after denervation. All animals except one rat treated with pargyline at the time of denervation lacked signs of general distress. The starts of the degeneration contraction were delayed in the rats given 200 mg/kg of par[illegible] gyline (see Table I). In all other groups, however, the MAO inhibitors [illegible] premature onset of the contraction. The contraction of these groups ended [illegible] normal time. In all treated groups, except in those given pargyline at [illegible] the slope of the ascending curve limb tended to be steeper than in the [illegible] the descending limb to be less steep. Thus, inhibition of MAO seems to [illegible] development and a slower disappearance of the degeneration contra[illegible]

TABLE I [illegible]

Treatment design	n	Time of start (T_{50s}) hours[1]	Time of end (T_{50d}) hours[1]	Rise time (T_{70s}—T_{30s}) hours	Fall time (T_{50d}—T_{70d}) hours
Pargyline 200 mg/kg at denervation	3	16.4, 16.4, 16.6 } 16.47	28.0, 27.1, 26.6 } 27.23	1.4, 1.3, 1.2 } 1.30	1.0, 0.8, 3.3 } 1.70
Pargyline 200 mg/kg at 10 hours[1]	3	15.9, 14.0, 15.8 } 15.23	23.1, 23.0, 25.8 } 23.97	0.8, 1.6, 1.0 } 1.13	0.7, 1.4, 1.3 } 1.13
Nialamide 200 mg/kg at denervation	5	14.04 ± 0.14	22.98 ± 0.69	0.74 ± 0.14	3.04 ± 0.90
Controls	12	14.95 ± 0.30	21.97 ± 0.39	1.27 ± 0.17	1.40 ± 0.31
Pargyline 100 mg/kg at denervation	5	14.23* ± 0.40	21.73 ± 0.38	1.12 ± 0.27	2.74 ± 1.21
Pargyline 100 mg/kg at 10 hours[1]	5	14.20* ± 0.20	23.32 ± 0.76	1.10* ± 0.22	2.41* ± 0.29
Nialamide 100 mg/kg at denervation	5	13.92** ± 0.32	24.70 ± 0.76	1.27 ± 0.08	2.27 ± 0.62
Phenprazine 10 mg/kg at denervation	5	14.13* ± 0.30	23.88 ± 0.71	1.42 ± 0.30	2.41* ± 0.24
Controls	15	15.66 ± 0.33	24.19 ± 0.29	1.78 ± 0.16	1.39 ± 0.25

[1] time of start and time of injection are expressed in hours after denervation

trally. The size of the contraction was increased in all treated groups when measured as area covered by the contraction curve, duration or maximal amplitude (height) (see Table II). Pargyline had a greater effect on the size of the contraction when given at the time of the denervation than when given at 10 hrs after denervation, which is 4 to 5 hrs before the start of the contraction.

Effects of reserpine injected at 12 hrs after denervation

Depletion induced by reserpine usually is not accompanied by any sympathomimetic actions because the transmitter is released onto MAO and reaches the neuroeffector space in deaminated form (for ref. see Iversen 1967). It appeared to be of interest to study the functional effects of reserpine given at the stage of degeneration just preceding that of the start of the degeneration transmitter release. Two rats operated as usual with one side denervated and the other decentralized were treated with 1 mg/kg of reserpine s.c. at 12 hrs after denervation which is 2 to 3 hrs before

Treatment design	n	Area covered by curve mm × hours	Duration (T_{50d}—T_{50a}) hours	Height (peak aperture on den side) mm	Aperture on decent side[a] mm
Pargyline 200 mg/kg at denervation	3	23 3 24 6 } 23 77 23 4	11 6 10 7 } 10 76 10 0	5 3 5 2 } 5 27 5 3	2 6 2 4 } 2 63 2 9
Pargyline 200 mg/kg at 10 hours[1]	3	21 3 22 0 } 19 90 16 4	7 2 9 0 } 8 73 10 0	5 3 4 8 } 5 30 5 8 ±	3 5 2 4 } 3 10 3 4
Nialamide 200 mg/kg at denervation	5	16 10±0 46 **	8 94±0 66 *	5 21±0 13 **	3 29±0 14 **
Controls	12	10 85±1 22	7 02±0 44	4 48±0 11	2 58±0 14
Pargyline 100 mg/kg at denervation	5	23 53±0 46 *** p<0 001	10 50±0 51 ** n s	5 24±0 068 *** n s.	3 18±0 068 * n s
Pargyline 100 mg/kg at 10 hours[1]	5	19 37±0 84 **	9 12±0 90	5 18±0 067 ***	3 04±0 11
Nialamide 100 mg/kg at denervation	5	25 63±2 17 ***	10 78±0 65 **	5 07±0 071 ***	2 56±0 16
Phenіprazine 10 mg/kg at denervation	5	24 40±0 83 ***	9 75±0 58	5 17±0 091 ***	2 65±0 13
Controls	15	14 60±0 82	8 53±0 30	4 31±0 11	2 67±0 10

[1] hours after denervation
[a] the size of palpebral aperture on the decentralized side when the degeneration contraction had its peak on the denervated side

the expected start of the degeneration contraction The drug did not induce any excitatory effect on any side and there was no degeneration contraction within 18 hrs after the injection

Inhibition of tyrosine hydroxylase by the methyl ester of l α methyltyrosine and the degeneration contraction

With the aim to test if there is any significant contribution of newly synthetized noradrenaline to the degeneration contraction rats were treated with the methylester of l α methyltyrosine (H 22/07) which is known to inhibit the synthesis of noradrenaline in vivo at the rate limiting step tyrosine to dopa (Spector, Sjordsma and Udenfriend 1965 Corrodi and Hansson 1966) The results are shown in Table III In one experiment 3 groups of rats (n—4—5) were given 200 mg/kg of H 22/07 i p.

TABLE III [illegible]

Treatment design	n	Time of start(T_{50a}) hours	Area covered by curve mm × hours	Duration (T_{50d}—T_{50a}) hours	Height (peak aperture on denervated side) mm
200 mg/kg at denervation	4	17 05±0 55	13 05±1 20	7 75±0 81	4 61±0 31
Saline	4	16 35±0 46	14 03±2 02	8 40±0 96	4 62±0 42
200 mg kg at 10 hours[1]	5	15 22±0 93	9 56±1 11	6 78±0 74	3 70±0 21
Saline	5	15 04±0 70	9 50±1 65	6 04±0 46	4 49±0 34
200 mg kg at 12 hours[1]	5	14 98±0 62	14 18±1 45	8 18± 091	4 61±0 39
Saline	4	14 28±0 28	17 09±0 45	7 88±0 88	4 47±0 091
250 mg kg at 12 hours[1]	5	15 40±0 51	12 58±1 50	8 68±0 62	4 35±0 23
Saline	5	15 44±0 18	15 29±1 07	9 30±0 38	4 61±0 61

[1] time of start and time of injection are expressed in hours after denervation

at the time of denervation and at 10 or 12 hrs after denervation respectively In another experiment 5 rats were treated with 250 mg/kg of H 22/07 s c at 12 hrs after denervation The control rats run simultaneously on each day of experiment were given 0 9 % NaCl instead of the drug From Table III it is evident that the inhibition of the synthesis of noradrenaline during the post denervation time interval did not influence the size of the degeneration contraction measured as area covered by the contraction curve duration or maximum amplitude (height) Neither did it influence the time course of the contraction

Discussion

Inhibition of monoamine oxidase (MAO) and the degeneration contraction

The postdenervation disappearance of noradrenaline from sympathetically innervated organs (Benmiloud and Euler 1963 Malmfors and Sachs 1965 Pluchino *et al* 1970) and the onset of the degeneration contraction of adrenergically innervated smooth muscles Langer 1966 Lundberg 1969 are delayed by the neuron blocking quarternary amines bretylium and beta TM 10 The mechanism of the delaying effect is unknown There seems to be no simple relation between the neuron blocking and the delaying action of bretylium Lundberg 1969 and 1970) Bretylium and beta-TM 10 are also known to inhibit MAO (for ref see Introduction) Since bretylium is accumulated in adrenergic neurons Boura *et al* 1960) it might block

intraaxonal MAO efficiently despite its relative low activity as enzyme inhibitor (Furchgott and Sanchez Garcia 1966 Clarke and Leach 1968) Therefore, it was necessary to test if MAO inhibition has a delaying effect In the present experiments 3 different MAO inhibitors nialamide phenipraziine and pargyline have been studied With the exception of very high doses of pargyline each drug started the degeneration contraction prematurely instead of delaying it Thus the MAO inhibitors did not block the degeneration transmitter release The finding also indicates that the delay induced by bretylium or beta TM 10 is not only caused by inhibition of MAO The present result is in accordance with the finding of Malmfors and Sachs (1965) who using their histochemical technique observed that nialamide at high doses did not obviously delay the disappearance of endogenous noradrenaline from the adrenergic nerves of the rat iris after denervation However, Hennemann and Trendelenburg (1970) have recently found that 100 mg/kg or pargyline injected at the time of denervation delayed the decline of endogenous noradrenaline from submaxillary glands of the rat as much as two doses of 50 mg/kg of beta TM 10 did when given at 6 and 12 hrs after denervation They felt that the delayed decline of noradrenaline induced by pargyline or beta TM 10 was due to inhibition of MAO Thus at first sight there seems to be a discrepancy between the effect of pargyline on the degeneration processes in the submaxillary gland and those in the periorbital smooth muscle However this could be due to the fact that besides from the difference in organs two different aspects of degeneration were studied, in the submaxillary gland experiments the disappearance of the transmitter from the whole organ was measured without any determination of functional effects whereas in the present experiments the mechanisms responsible for the onset of the degeneration contraction were studied Maybe pargyline delays the decline of noradrenaline in the gland by inhibiting the extra neuronal MAO which in this organ represents about two thirds of the total amount of the enzyme (Almgren *et al* 1966) This would improve the conditions for the released transmitter to be preserved by binding to extra neuronal sites for instance by an Uptake₂ (Almgren Anden and Waldeck 1965 Iversen 1[illegible]7 Lightman and Iversen 1969)

It has been found in earli[illegible]dies on the degeneration contraction in the rat that BW 392C60 which is a guanidine [illegible]ative with neuron blocking ability and distinctly a higher MAO inhibiting activity than [illegible]um (Kuntzman and Jacobson 1963) lacks delaying effect (Lundberg 1969) This fact also favours the view that the delaying effect of bretylium is not linked to inhibition of MAO

Besides the fact that they tended to induce a premature start of the degeneration contraction the MAO inhibitors used in this work increased the size of the contraction These effects of the drugs which belong to two structurally different groups of MAO inhibiting agents are probably due to inhibition of MAO Pargyline influenced the course and the size of the contraction significantly even when given at 10 hrs after the denervation which is 4 to 5 hrs before the normal start of the contraction Thus the effects appear mainly to be due to inhibition of MAO around the start and during the course of the degeneration transmitter release However pargyline in

creased the size of the contraction more when injected at the time of denervation than when given at 10 hrs after the operation This suggests that preservation of noradrenaline caused by the inhibition of MAO and increased amount of transmitter available for the degeneration release is responsible for some part of the effect. The present results indicate that during the normal degeneration release from the intraaxonal storage sites part of the transmitter content is deaminated by intraaxonal MAO Maybe at the beginning of the release the noradrenaline is mostly broken down and no contraction can be observed until a critical rate of release is reached Recently Obianwu (1969) showed that inhibition of MAO by pargyline at 75 mg/kg did not potentiate the contraction of the rat lower eyelid to nerve stimulation Thus the mechanisms of transmitter release induced by degeneration processes and nerve stimulation differ This fact and the earlier findings suggesting that the delaying effect of bretylium is probably not due to a neuron block (Lundberg 1970) appear to rule out the possibility that the degeneration transmitter release is caused by stimulation from an irritative lesion at the cut end of the nerve

Effect of reserpine

Reserpine is thought to destroy granular binding sites of noradrenaline in normal nerves thereby exposing the stored transmitter to the intraaxonal MAO which inactivates it before it leaks into the neuro effector space (for ref, see Iversen 1967) Langer (1966) showed that no degeneration contraction of the cat nictitating membrane occurred after treatment with reserpine (0 1 mg/kg s c) injected at the time of denervation and again 24 hrs later The similar finding was obtained in the present study where 1 mg/kg of reserpine was given to rats at 12 hrs after denervation Reserpine abolished the degeneration contraction expected to start 2 to 3 hrs later without inducing any sympathomimetic action Within 4 to 8 hrs after an i p injection of reserpine at 1 mg/kg the adrenergic nerves in normal rat iris are almost completely depleted of noradrenaline (Malmfors 1965) The present results indicate that reserpine causes a rapid inactivation of the noradrenaline stored in the nerve endings even at the stage of degeneration just preceding or including the start of the normal degeneration transmitter release This suggests that MAO at this stage of degeneration is still functionally active and that the normal degeneration release is not only induced by a reserpine like destruction of granular binding sites of the transmitter Somehow a considerable part of the transmitter released by degeneration escapes the MAO

Inhibition of tyrosine hydroxylase and the degeneration contraction

The noradrenaline content in intact adrenergic nerves is continuously kept at a constant level This is probably achieved by a feedback mechanism in which increased levels of noradrenaline inhibit synthesis Possibly there is also increased enzymatic breakdown of the transmitter (for ref see Iversen 1967) A change in this balance e g by unopposed new-synthesis of transmitter at a certain stage of degeneration could be responsible for the considerable intensity and duration of the de

generation contraction In the present experiments the methylester of l α methyl tyrosine (H 22/07) which inhibits the synthesis of noradrenaline at the rate limiting step tyrosine to dopa (Spector *et al* 1965 Corrodi and Hansson 1966) did not significantly diminish the size of the degeneration contraction This was so both when the drug was given at the time of denervation or at 10 or 12 hrs later Conceivably, at the time of the late injections the function of an axonal amino-acid pump could have deteriorated This would prevent the drug from reaching enzyme blocking concentrations in the nerves But, since the duration of the synthesis inhibition induced by the drug exceeds at least 16 hrs (Corrodi and Hansson 1966 Corrodi and Fuxe 1967) the results appear none the less to rule out any significant contribution of recently formed transmitter to the degeneration contraction

I want to thank Professor E Barány for unfailing encouragement and advice Professor L [illegible] stimulating and in [illegible] e drug H 22/07, [illegible] k Forskning and [illegible] edical Research Council

References

ALMGREN O N E ANDEN J JONASON K A NORBERG and L OLSSON Cellular localization of monoamine oxidase in salivary glands *Acta physiol scand* 1966 *67* 21—26
ALMGREN O N E ANDÉN and B WALDECK Extraneuronal binding as a possible factor of tyramine uptake by sympathetic nerves *Life Sci* 1965 *4* 121—126
BENMILOUD M and U S v EULER Effects of bretylium reserpine guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland *Acta physiol scand* 1963 *59* 34—42
BOURA A L A F C COPP W G DUNCOMBE A F GREEN and MCCOUBREY The selective accumulation of bretylium in sympathetic ganglia and their postganglionic nerves *Brit J Pharmacol* 1960 *15* 265—270
CLARKE D E and G D H LEACH The influence of bretylium on the interactions of infused sympathomimetic amines and tyramine in the reserpine treated pithed rat *Brit J Pharmacol* 1968 *32* 392—401
CORRODI H and K FUXE The effect of catecholamine precursors and monoamine oxidase inhibition on the amine levels of central catecholamine neurons after reserpine treatment or tyrosine hydroxylase inhibition. *Life Sci* 1967 *6* 1345—1350
CORRODI H and L C F HANSSON Central effects of an inhibitor of tyrosine hydroxylation *Psychopharmacologia* (Berl) 1966 *10* 116—125
EMMELIN N and B STROMBLAD A transient period c secretion from the parotid gland after post ganglionic parasympathetic denervation *J Physiol* Lond) 1957 *140* 21—22P
FURCHGOTT R F and P SANCHEZ GARCIA Inhibition of monoamine oxidase of adrenergic nerve terminals by bretylium *Pharmacologist* 1966 8 176
GIACHETTI A and P A SHORE Monoamine oxidase inhibition in the adrenergic neuron by bretylium debrisoquin and other adrenergic neuronal blocking agents *Biochem Pharmacol* 1967 *16* 237—238
HENNEMAN H M AND L TRENDELENBURG Effect of the adrenergic neurone blocker beta TM 10 on the depletion of noradrenaline induced by denervation or reserpine *Naunyn-Schmiedeberg s Arch exp Path Pharmak* 1970 *265* 363—371
IVERSEN L L *The uptake and storage of noradrenaline in sympathetic nerves* Cambridge University Press 1967
KUNTZMAN R and M M JACOBSON Monoamine oxidase inhibition by a series of compounds structurally related to bretylium and guanethidine *J Pharmacol exp Ther* 1963 *141* 166—172
LANGER S Z The degeneration contraction of the nictitating membrane in the unanesthetized cat *J Pharmacol exp Ther* 1966 *151* 66—72

LIGHTMAN S L and L L IVERSEN The role of uptake2 in the extraneuronal metabolism of catecholamines in the isolated rat heart *Brit J Pharmacol* 1969 *37* 638—649

LUNDBERG D Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat *Acta physiol scand* 1969 *75* 415—426

LUNDBERG D Bretylium and the degeneration contraction of the sympathetically innervated periorbital smooth muscle in the rat *Acta physiol scand* 1970 *79* 411—422

MALMFORS T Studies on adrenergic nerves *Acta physiol scand* 1965 *65* Suppl 248

MALMFORS T and C SACHS Direct studies on the disappearance of the transmitter and changes in the uptake storage mechanism of degenerating adrenergic nerves *Acta physiol scand* 1965 *64* 211—223

MCCOUBREY, A Biochemical properties of bretylium *J Pharm Pharmacol* 1962 *14* 727—734

OBIANWU H O Possible functional differentiation between the stores from which adrenergic nerve stimulation tyramine and amphetamine release noradrenaline *Acta physiol scand* 1969 *75* 92—101

PLUCHINO S L S VAN ORDEN III, P R DRASKOCZY S Z LANGER and U TRENDELENBURG The effect of beta TM 10 on the pharmacological biochemical and morphological changes induced by denervation of the nictitating membrane of the cat *J Pharmacol exp Ther* 1970 In press

SEARS M L and E H BARANY Outflow resistance and adrenergic mechanisms *Arch Ophthal* 1960 *64* 839—848

SPECTOR S SJOERDSMA A and S UDENFRIEND Blockade of endogenous norepinephrine synthesis by alfa methyl tyrosine an inhibitor of tyrosine hydroxylase *J Pharmacol exp Ther* 1965 *147* 86—95

TREISTER G and E H BARANY The effect of bretylium on the degeneration mydriasis and intraocular pressure decrease in the conscious rabbit after unilateral cervical ganglionectomy *Invest Opthal* 1970 In press

Acta physiol. scand. 1970. 80. 117—121

From the Institute of Medical Physiology C, University of Copenhagen, Denmark

The Effect of Dinitrophenol on Secretory Potentials, Secretion and Potassium Accumulation in the Perfused Cat Submandibular Gland

By

OLE HOLGER PETERSEN

Received 13 February 1970

Abstract

PETERSEN O H *The effect of dinitrophenol on secretory potentials, secretion and potassium accumulation in the perfused cat submandibular gland* Acta physiol scand 1970 *80* 117—121

Dinitrophenol strongly depresses salivary secretion and potassium uptake after acetylcholine-induced potassium loss in the perfused [illegible]
secretory [illegible]
change [illegible]
potentials [illegible]
potential [illegible]

Lundberg (1955, 1957 a, b and c) proposed that the hyperpolarization of the basal acinar cell membrane (secretory potential) occurring during stimulation of the submandibular or sublingual gland was caused by an active inward transport of chloride ions However, it was recently shown that the secretory potentials were uninfluenced by perfusion with a sulphate Locke solution that did neither contain chloride nor bicarbonate (Petersen and Poulsen 1968 and 1969) The most ready explanation to account for the secretory potential would be that acetylcholine liberated from the postganglionic nerve endings acts on the basal acinar cell membrane by increasing the permeability to potassium allowing this ion to diffuse down its electrochemical gradient out of the acinar cells If this were the correct explanation the secretory potentials should not be directly dependent on energy metabolism In the present work it is shown that dinitrophenol (DNP) in a concentration sufficient to inhibit secretion of saliva markedly has very little influence on the secretory potentials

Methods

Cats anesthetized with chloralose (80 mg/kg i p) were used The preparation of the submandibular gland for artificial perfusion has been described previously (Petersen and Poulsen 1967 a) The perfusion fluid used contained (mM) NaCl 140, KCl 4 0 NaH_2PO_4 0 6 Na_2HPO_4 2 4 $CaCl_2$ 1 5 $MgCl_2$ 1 0, glucose 5 5 2 4 dinitrophenol (DNP) was added

Fig 1 This figure should be read from right to left A deflection downwards denotes increased negativity of the microelectrode The recordings have been slightly retouched to improve the contrast The sudden jump in potential seen in all recordings corresponds to the insertion of the microelectrode into an acinus A) The upper part shows a resting and a secretory potential recorded during perfusion with control Locke solution The lower part shows a resting and a secretory potential recorded from the same experiment 17 min after shift to DNP Locke solution ↑ denotes injection of 1 μg ACh B) The upper part shows a resting and 2 secretory potentials recorded 19 min after start of perfusion with DNP Locke solution The lower part shows a resting and 3 secretory potentials recorded from the same experiment 27 min after shift to control Locke solution ↑ denotes injection of 0 1 μg ACh

obtain a final concentration of 10^{-4} M The perfusion fluids were equilibrated with pure oxygen Perfusion was carried out at room temperature

Membrane potentials were measured with conventional glass capillary micropipettes filled [illegible] was [illegible] 8) [illegible] of [illegible] ter W P instruments model M 4 and the signal was thereafter led to a d c amplifier (Tektronix type 2A63 displayed on an oscilloscope screen (Tektronix type 565) and recorded on an UV recorder S E 3006 An indifferent Ag AgCl electrode was placed on the surface of the gland

In some experiments the concentration of potassium in the fluid coming out of the gland vein was measured using an Eppendorff flame photometer

The gland was stimulated by rapid i a injections of acetylcholine (ACh or adrenaline The dimensions of the submandibular duct cannula used were such that 1 drop of saliva corresponded to 35 μl

Results

In Fig 1 are shown typical recordings of transmembrane resting and secretory potentials It is seen that the amplitude of the secretory potentials are uninfluenced by the presence of dinitrophenol In Fig 2 is shown the course of one experiment The total material (6 expts on 6 cats is given in Fig 3 All the secretory potentials recorded during perfusion with DNP were obtained after the gland had been perfused for at least 15 min with the inhibitor As seen in Fig 3 the sizes of the secretory potentials recorded after injection of 0 1 μg ACh did not differ much from those recorded after injection of 1 μg ACh The duration of the secretory potentials seemed however, to depend on the dose of ACh used Injection of 1 μg ACh evoked secretory potentials

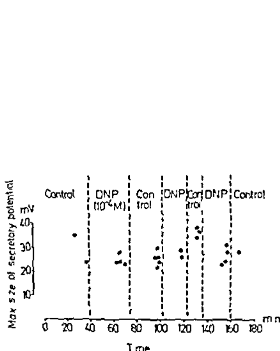


Fig 2

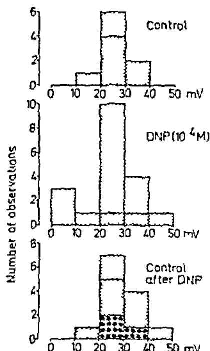


Fig 3

Fig 2 Maximal sizes of secretory potentials (the difference between the maximal membrane potential after stimulation and the resting membrane potential), obtained after intraarterial injections of 0 1 μg ACh, plotted as a function of time after start of perfusion

Fig 3 Distribution of the maximal sizes of the secretory potentials obtained during control periods periods of perfusion with DNP and control periods following after periods of inhibition. ☐ 0 1 μg ACh, ☐ 1 μg ACh and ☐ 1 μg adrenaline The data have been pooled from 6 expts on 6 cats

with a duration of 21 sec ± 2 (S E) (n = 5) (control) and 19 sec ± 4 (n = 4) (DNP) whereas injection of 0 1 μg ACh evoked secretory potentials with a duration of 13 sec ± 1 (n = 9) (control) and 9 sec ± 1 (n = 11) (DNP) The flow of perfusion fluid through the gland immediately after an injection of ACh was 8 5 ml/min ± 1 5 (n = 6) (control) and 8 7 ml/min ± 1 1 (n = 9) (DNP) The sizes of the resting membrane potentials were 22 mV ± 1 (n = 13) in the control periods, 22 mV ± 1 (n = 16 during perfusion with DNP Locke solution and 22 mV ± 1 (n = 20) in the control periods following after periods of DNP perfusion

Perfusion of the cat submandibular gland with DNP Locke solution always inhibited secretion of saliva severely 0 1 μg ACh which was most often used to evoke secretory potentials only produced a scanty secretion that could hardly be evaluated but in 9 different glands where secretion of saliva was evoked after i a injections of 10 μg ACh the mean value of the number of drops of saliva secreted after each injection of ACh was 6 3 ± 1 2 during the control period 1 0 + 0 3 15 min after start of perfusion with DNP Locke solution (the first injection of ACh in the DNP period) and 4 1 ± 0 7 10 min after reintroduction of the control Locke solution

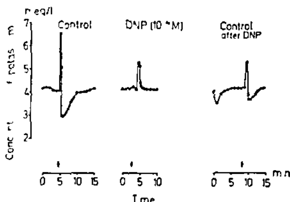


Fig 4 Concentration of potassium in the perfusion fluid coming out of the submandibular gland vein plotted as a function of time ↑ denotes injection of 10 μg ACh. In the period of DNP perfusion the injection of ACh was given 15 min after start of perfusion with DNP. Time zero in the last control period corresponds to the time of reintroduction of the control Locke solution

Reuptake of potassium into the gland after ACh induced loss of potassium was also severely inhibited during perfusion with DNP Locke solution. In Fig 4 is shown a typical experiment. It is seen that accumulation of potassium was abolished during perfusion with DNP Locke solution. This pattern was seen in all the 7 expts of this kind carried out

Discussion

In the present experiments it was shown that the size of the secretory potentials from acinar cells in the cat submandibular gland is uninfluenced by the presence of the metabolic inhibitor DNP in a concentration sufficient to inhibit secretion of saliva severely and to abolish reuptake of potassium after ACh induced potassium loss. Previously Petersen and Poulsen (1967 b) were able to show a very marked inhibition of the secretory potentials from the same gland using the same concentration of the inhibitor after much shorter periods of perfusion with the inhibitor. Petersen and Poulsen (1967 b) included in their material secretory pottentials evoked after electrical stimulation of the chorda lingual nerve. In the present work only stimulation with intraarterial injections of ACh was used. This is without doubt more reliable because one does not need to care whether nervous transmission between pre and postganglionic parasympathetic fibres has been blocked or not or whether release of ACh from the postganglionic nerve endings has been inhibited by DNP. Petersen and Poulsen (1967 b) stimulated the gland many times during each period of perfusion with DNP whereas in the present work care was taken only to stimulate the gland 5 times during each period of perfusion with DNP Locke solution. Also the doses of ACh used were much smaller in the present work than in the previous one. Nevertheless the sizes of the secretory potentials were greater in the present work. These differences may have been of significance since it has been shown (Petersen 1970) that reuptake of potassium into the cat submandibular gland after ACh induced potassium loss was abolished during perfusion with DNP Locke solution. This finding was also made in the present work (Fig 4). How great a part of the ACh induced potassium loss that originates from the acinar cells is unknown

(Petersen 1970) However it is probable that the acinar cells in the paper of Petersen and Poulsen (1967 b) were somewhat depleted of potassium because the sizes of the resting membrane potentials recorded during perfusion with metabolic inhibitors were smaller than those recorded during control periods In the present work the sizes of the resting potentials were the same in the material collected during control periods and periods of perfusion with DNP Locke solution

It is difficult to explain why the duration of the secretory potentials recorded during periods of perfusion with DNP Locke solution was smaller than the duration of those recorded during the control periods Possibly DNP changes the electrical properties of the cell membranes It is known that DNP (5×10^{-5} M) can cause a depression of the ion permeability of the membrane junctions between chironomus salivary gland cells (Politoff Socolar and Loewenstein 1969) Whether such an effect occurs in the gland studied in this work is unknown and it is even difficult to tell whether such an effect would influence the secretory potentials

The results of the present experiments indicate that the secretory potentials are not directly dependent on energy metabolism Therefore the mechanism of the secretory potentials is probably an enhanced permeability of the basal acinar cell membrane to one or more ions As it has been shown that the sizes of the secretory potentials are diminished when the extracellular potassium concentration is increased (Yoshimura and Imai 1967 and Petersen and Poulsen 1967 a) it seems likely that an enhanced permeability of the basal acinar cell membrane to potassium could be the underlying mechanism of the secretory potential

References

EIDE E and Y KALLSTROM Remotely controlled micromanipulator for neurophysiological use *Acta physiol scand* 1968 *73* 2A—3A

LUNDBERG A The electrophysiology of the submaxillary gland of the cat *Acta physiol scand* 1955 *35* 1—25

LUNDBERG A Secretory potentials in the sublingual gland of the cat *Acta physiol scand* 1957a *40* 21—34

LUNDBERG A The mechanism of establishment of secretory potentials in sublingual gland cells *Acta physiol scand* 1957b *40* 35—58

LUNDBERG A Anionic dependence of secretion and secretory potentials in the perfused sublingual gland *Acta physiol scand* 1957c *40* 101—112

PETERSEN O H Some factors influencing stimulation induced release of potassium from the cat submandibular gland to fluid perfused through the gland *J Physiol* (Lond) 1970 *208* 431—447

PETERSEN O H and J H POULSEN The effects of varying the extracellular potassium concentration on the secretory rate and on resting and secretory potentials in the perfused cat submandibular gland *Acta physiol scand* 1967a *70* 293—298

PETERSEN O H and J H POULSEN Inhibition of salivary secretion and secretory potentials by g strophanthin dinitrophenol and cyanide *Acta physiol scand* 1967b *71* 194—202

PETERSEN O H and J H POULSEN Secretory potentials potassium transport and secretion in the cat submandibular gland during perfusion with sulphate Locke s solution *Experientia* (Basel) 1968 *24* 919—920

PETERSEN O H and J H POULSEN Transmembrane secretory potentials and electrolyte transients in salivary glands In *Exocrine Glands* A satellite symposium of the XXIV International Congress of physiological sciences Eds Botelho S Y F P Brooks and W B Shelley University of Pennsylvania Press Philadelphia 1969 pp 3—20

POLITOFF A L S J SOCOLAR and W R LOEWENSTEIN Permeability of a cell membrane junction Dependence on energy metabolism *J gen Physiol* 1969 *53* 498—515

YOSHIMURA H and Y IMAI Studies on the secretory potential of acinal cell of dog s submaxillary gland and the

Acta physiol. scand. 1970. 80. 122—130

From the Institute of Physiology, University of Aarhus, Denmark

Exchange of Glucose between Plasma, Brain Extracellular Fluid and Cerebral Ventricles in Cats and Effects of Intraventricular Acetazolamide and Insulin

By

H E Brondsted

Received 16 February 1970

Abstract

Brondsted H E *Exchange of glucose between plasma, brain extracellular fluid and cerebral ventricles in cats and effects of intraventricular acetazolamide and insulin* Acta physiol scand 1970 *80* 122—130

The ventriculo aqueductal perfusion technique was used for a quantitative study of the transport of glucose between the cerebral ventricles and surrounding tissues in cats The inflow perfusion fluid contained U-^{14}C-D glucose and unlabelled D glucose Carrier systems appeared to operate symmetrically Intraventricular acetazolamide or insulin did not influence the transport of glucose in the brain At zero net flux of glucose in the system the concentration of glucose in brain extracellular fluid was estimated to be the same as in the ventricles i e about 40 per cent below the concentration in arterial plasma water

Previous investigations on the anesthetized cat (Brondsted 1970a and b) have shown the presence of a carrier mediated unidirectional flux of glucose from the cerebral ventricles to surrounding tissues Ouabain intraventricularly administered (5× 10^{-5} M partly inhibited this flux while the tissues lost potassium and gained sodium and chloride At normal ventricular concentration of glucose (3 5 mM) about 40 per cent of total unidirectional transport of glucose from the ventricles occurred by simple diffusion

In the experiments to be described further information about the transport system has been obtained The fluid perfusing the first three cerebral ventricles contained U^{14}C-D glucose in order to determine the unidirectional fluxes of glucose between the ventricles and surrounding tissues during steady state The following has been investigated the *unidirectional fluxes of glucose from blood and brain into the ventricles* at various concentrations of glucose in plasma the effect of acetazolamide (diamox) intraventricularly upon the fluxes of glucose upon cerebrospinal fluid (CSF) formation and upon net fluxes of chloride and potassium, the effect of intraventricular insulin upon glucose fluxes and upon the absorption of ^{14}C D galactose

from the ventricles Finally, the concentration of glucose in brain extracellular fluid has been estimated from the concentrations of glucose in plasma water and ventricular CSF at zero net flux of glucose in the system.

Methods

Cats of either sex weighing from 3—5 kg were anesthetized with chloralose 70 mg/kg administered i.p as a 1 % solution. Small doses of a 6 % solution of pentobarbital sodium were given i.p as necessary Tracheotomy with intubation was carried out and a polythene catheter was inserted through the right femoral artery into the abdominal aorta The catheter served for blood sampling and for monitoring of mean arterial blood pressure by means of a mercury manometer Rectal temperature was maintained at 37.5—38.5° C.

The procedures used to establish the perfusion system, the composition of the inflow solution and the analytical techniques have been described previously (Brøndsted 1970a and b) The inflow solution contained D glucose in various concentrations (see *Results*) and U-^{14}C D glucose (5 μC per 100 ml solution) The rate of inflow was 110 μl/min (S D = 0 8 n = 72 and one half of this flow was diverted to each of the lateral ventricles In experiments with insulin the rate of inflow was slower (see *Results*) and only one lateral ventricle was cannulated. Effluent samples were collected from the aqueductal catheter during successive periods of 15 min. Effluent volumes were determined by weighing to the nearest 0 1 mg Only effluent samples which were clear and free from blood were used for analyses. The rate of respiration pulse rate reflexes etc. were controlled regularly Materials and instruments were sterilized before coming into contact with tissues or fluids containing glucose

Analytical Samples of arterial blood were taken every half hour, and glucose was determined in plasma or in whole blood and in effluent samples (all in duplicate) using a glucose oxidase method. The concentration of glucose in plasma water was found by multiplication of the concentration in plasma by 1 03 (Brøndsted 1970b) ^{14}C-activity in the effluent was determined using a Packard Tri-Carb liquid scintillation system. Potassium in the effluent was determined using an Eppendorf flame photometer and chloride by electrometric titration.

Computations These were as described (Brøndsted 1970a and b In short the unidirectional flux of ^{14}C-D-glucose from the cerebral ventricles into plasma and brain ($J^*_{v\,pb}$ cpm/min) was computed as the rate of inflow of tracer minus the rate of outflow of tracer The unidirectional flux of glucose from the ventricles ($J_{v\,pb}$ μmoles min was derived on the following assumption

$$J_{v\,pb}/\overline{C_v} = J^*_{v\,pb}/\overline{C^*_v}$$

where $\overline{C_v}$ (mM) is the mean ventricular concentration of glucose the arithmetical mean of the concentration in the inflow and in the outflow and $\overline{C^*_v}$ cpm/ml is the mean ventricular concentration of ^{14} C-D-glucose assuming an exponential decrease of concentration within the ventricles $\overline{C^*_v}$ = 0 37 times inflow concentration + 0 63 times outflow concentration

The net flux of glucose between the ventricles and surrounding tissues was determined as the rate of outflow of glucose minus the rate of inflow of glucose Similar calculations were used for potassium and chloride Net fluxes were called positive if they were directed from the tissues into the ventricles The unidirectional flux of glucose from blood and brain into the ventricles ($J_{pb\,v}$ μmoles/min was determined as the sum of the net flux and the unidirectional flux from the ventricles

Steady state After 45—60 min of perfusion the ^{14}C-D glucose in the effluent remained constant within 1.9 per cent

Results

Transport of glucose from blood to cerebral ventricles In Fig 1 are seen the magnitudes of the unidirectional fluxes of glucose into the cerebral ventricles at different concentrations of glucose in arterial plasma water Each point represents one experiment where plasma glucose levels were stable within 3 3 per cent for 1/2—2 1/2 hrs The flux of glucose was determined from one or two 15 min sampling periods during steady state perfusion towards the end of the period with stable plasma gluco

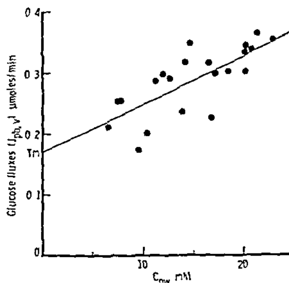


Fig. 1. Unidirectional fluxes of glucose from blood and brain into the perfused cerebral ventricles (ordinate, $J_{pb,v}$). In each experiment there were stable concentrations of glucose in arterial plasma water (abscissa, C_{pw}). The straight line was computed by the method of least squares: $y = (0.0080 \pm 0.0014) \times + (0.170 \pm 0.022)$. The slope defines a transfer constant for simple, unidirectional diffusion of glucose; it is significantly different from zero ($p < 0.001$). The intercept with the y-axis, Tm = 0.17, defines transport maximum for total unidirectional transport minus simple diffusion; this value too is significantly different from zero, $p < 0.001$.

values. The transport appeared to be linearly related to plasma glucose concentrations with a slope of 0.008 ml/min. It suggests that there is a passive as well as an "active" component of the transport of glucose. The intercept with the ordinate defines transport maximum (Tm) for total unidirectional transport minus simple diffusion (equal to "active" transport).

Acetazolamide intraventricularly. After a control period, acetazolamide was added in a small volume to the inflow perfusion fluid so as to give a final concentration of 10^{-3} M. The results in Table I are from an experiment which is representative for two experiments. Mean values from twelve 15-min sampling periods during steady state were used, six from the control period and six from the experimental period. The rate of net CSF formation decreased to 40—50 per cent. This effect was always seen during the first 15-min sampling period; it was highly significant (t-test, $p <$

TABLE I. Acetazolamide in the inflow (10^{-3} M) after a control period without acetazolamide: unidirectional flux of glucose from the cerebral ventricles ($J_{v,pb}$) and from plasma and brain ($J_{pb,v}$) is shown together with the concentration of glucose within the ventricles (C_v) and in plasma water (C_{pw}), respectively, rate of net CSF formation with net flux of chloride from the tissues into the perfusate, and net flux of potassium.

		Control	Acetazolamide
$J_{v,pb}$	µmoles/min	0.16	0.15
C_v	mM	6.1	6.1
$J_{pb,v}$	µmoles/min	0.29	0.28
C_{pw}	mM	19.7	18.3
Net CSF	µl/min	6.0	2.4
Net Cl	µeq/min	1.2	1.0
Net K^+	µeq/min	0	0

TABLE II Insulin in the inflow (0 1 I U /ml) and slow rates of perfusion (1/30 ml/min) Symbols are the same as in Table I The absorption of ¹⁴C-D galactose (exp no 5) is presented as per cent of rate of inflow

	Control		Insulin	
Expt no	$J_{v\ pb}$ (μmoles min)	$\overline{C}_v$ (mM)	$J_{v\ pb}$ (μmoles/min)	$\overline{C}_v$ (mM)
1	0 04	4 0	0 04	4 1
2	0 06	4 3	0 07	4 4
3	0 07	5 0	0 07	5 1
4	0 11	12 2	0 11	12 2
	^{14}C-gal		^{14}C-gal	
5	24 5 %	4 6	22 8 %	4 5

0 005) whereas the small decrease seen in the net flux of chloride was insignificant The unidirectional fluxes of glucose between the cerebral ventricles and surrounding tissues and the net flux of potassium remained unchanged

Insulin intraventricularly Preliminary experiments with insulin in the inflow and a rate of perfusion of 0 11 ml/min showed no effect upon the absorption of glucose from the cerebral ventricles In order to obtain higher absorption values, slow rates of perfusion were used After a control period, glucagon free insulin was added in a small volume to the inflow perfusion fluid The results in Table II are mean values from 15 min sampling periods during steady state (control periods 2—5 sampling periods, and insulin periods 2—10 sampling periods) Insulin was present intraventricularly for 30—180 min and the total amount administered in this way corresponded to 0 1—0 3 I U Various concentrations of glucose intraventricularly were established In 1 expt (no 5) the inflow contained D galactose (5 6 mM) and ^{14}C D galactose instead of ^{14}C-D glucose No effects of insulin were observed upon the absorption of glucose or ^{14}C D galactose

It was thought that a possible small effect of insulin upon the transport of glucose might be disclosed by determination of the specific activity of effluent glucose after confinement of ^{14}C D glucose glucose and insulin within the cerebral ventricles for 1/2 hr After a period of steady state perfusion (45 min) the perfusion was stopped and the aqueductal catheter was closed by inserting a small glass stopper (Draskoci *et al* 1960) A small volume of the inflow solution (50—200 μl) was slowly injected into the ventricles, and half an hour later the aqueductal catheter was opened and the specific activity of effluent glucose determined ($SA_0 = C_0/C_0$ cpm/μmole) This represented the control values shown in Table III A period of steady state perfusion was re established (45 min) and the procedures were repeated with insulin in the same amount of solution as previously injected Various amounts of insulin were used No effects were observed upon blood sugar values or upon the specific activity of glucose in the effluent

ventricles and surrounding tissues was not influenced by insulin administered intraventricularly in such a way that insulin could not escape via the arachnoid villi into the blood The transport of glucose was followed for up to 3 hrs during insulin treatment and the concentration of glucose in the cerebral ventricles and in the blood were stable (Table III), *i e* the ventricular insulin did not indirectly influence the blood glucose values either Insulin (molecular weight at least 12,000) most probably had access to the parenchymal cells, as much larger molecules like peroxidase are able to penetrate from the ventricles into brain extracellular space (Brightman and Reese 1969) The carrier system for transport of glucose from the ventricles becomes saturated at ventricular concentrations above 10 mM (Brondsted 1970a), such concentrations were only established in 1 expt (no 4) so if an action of insulin was dependent upon an unsaturated carrier mechanism all requirements for an effect of insulin upon brain parenchymal cells were met in the other experiments Nor was the absorption of ^{14}C D galactose altered by such an administration of insulin (this monosaccharide is very poorly metabolized in cerebral tissues (Maddock, Hawkins and Holmes 1939))

There are some conflicting reports on the action of insulin administered intracranially Results from such experiments (all on dogs some of them after previous vagotomy) are obscured by the fact that insulin may have passed from the cisterna magna into the blood thereby lowering the C S F glucose level via a peripheral effect (Vuylsteke 1949 Leusen and Demeester 1949 Slovter and Sakata 1963 Chowers and Halpern 1966 Margolis and Altszuler 1968) The possibility that insulin in the present experiments may have caused an increase in brain glycogen content was not investigated such an effect has recently been reported by Mellerup and Rafaelsen (1969) who injected insulin into the cisterna magna of rats

Brain extracellular fluid (ECF) concentration of glucose With the glucose oxidase method the distribution ratio of 0 84 for glucose CSF/arterial whole blood in the anesthetized cat (Table IV) is the same as for CSF/capillary whole blood in human beings (0 86 Brondsted 1970c) and the ratio CSF/plasma of 0 58 in the cat is the same as for rabbits (0 57 Bradbury and Davson 1964) Table V shows that at approximately the same distribution ratios the net flux of glucose between the cerebral ventricles and surrounding tissues is near zero As there is no net flux between the ventricles and brain extracellular space (ECS) and as simple diffusion seems to be an important factor for glucose exchange at this site (Brondsted 1970a) the concentration of glucose in the two compartments must be equal In other words *when the concentration of glucose in the ventricles is about 40 per cent below that of plasma the concentration of glucose between brain parenchymal cells* (at any rate those surrounding the ventricles) *is also about 40 per cent below the concentration in plasma*

Bito and Davson (1966) found a distribution ratio of glucose CSF/arterial plasma about 73 per cent in the dog and they concluded similarly that CSF glucose was probably in equilibrium with ECF glucose at this ratio

A ratio ECF/blood glucose can be computed from reported measurements of rat

brain glucose, carried out under optimal conditions (rapid freezing at low temperatures *in situ*) the figures of Mark, Godin and Mandel (1968) gives a ratio ECF/whole blood glucose of 0 93, if it is assumed that brain intracellular *free* glucose is zero (Gey 1956) and if ECS is 15 per cent of tissue volume (Woodward Reed and Woodbury 1967, Cutler, Lorenzo and Barlow 1968) Similar ratios close to one can be computed from values reported by Flock Tyce and Owen (1966) and the control values from Stewart *et al* (1967) gives a ratio of 0 65 for ECF/serum glucose, in this case the rats r— it decapitated before freezing These ratios are in concordance with the ratios in Tab IV and V and suggest that under the experimental conditions the great majority, if not all of brain glucose is localized in an ECS with a size about 15 per cent and that ECF glucose is near equilibrium with ventricular glucose

This work was supported by a grant from Statens Almindelige Videnskabsfond

References

AMES A K HIGASHI and F B NESBETT Effects of PCO_2 acetazolamide and ouabain on volume and composition of choroid plexus fluid *J Physiol* (Lond) 1965 *181* 516—524

ATKINSON A J and M F WEISS Kinetics of blood-cerebrospinal fluid glucose transfer in the normal dog *Amer J Physiol* 1969 *216* 1120—1126

BITO L Z and H DAVSON Local variations in cerebrospinal fluid composition and its relationship to the composition of the extracellular fluid of the cortex *Exp Neurol* 1966 *14* 264—280

BRADBURY M W B and H DAVSON The transport of urea creatinine and certain monosaccharides between blood and fluid perfusing the cerebral ventricular system of rabbits *J Physiol* (Lond) 1964 *170* 195—211

B

B

E

E

C

glucose level of the blood and the cerebrospinal fluid in vagotomized dogs *Exp Neurol* 1966 *14* 383—389

CSERR H Potassium exchange between cerebrospinal fluid plasma and brain *Amer J Physiol* 1965 *209* 1219—1226

CUTLER R W P A V LORENZO and C F BARLOW Sulfate and iodide concentration in brain *Arch Neurol* (Chic) 1968 *18* 316—323

DAVSON H and C P LUCK The effect of acetazoleamide on the chemical composition of the aqueous humour and cerebrospinal fluid of some mammalian species and on the rate of turnover of ^{24}Na in these fluids *J Physiol* (Lond) 1957 *137* 279—293

DOMER F R Effects of diuretics on cerebrospinal fluid formation and potassium movement *Exp Neurol* 1969 *24* 54—64

DRAŠKOCI M W FELDBERG K FLEISCHHAUER and P S R K HARANATH Absorption of histamine into the blood stream on perfusion of the cerebral ventricles and its uptake by brain tissue *J Physiol* (Lond) 1960 *150* 50—66

FISHMAN R A Carrier transport of glucose between blood and cerebrospinal fluid *Amer J Physiol* 1964 *206* 836—844

FLOCK E V G M TYCE and C A OWEN Utilization of U-[^{14}C] glucose in brain after total hepatectomy in the rat *J Neurochem* 1966 *13* 1389—1406

GEY K F The concentration of glucose in rat tissues *Biochem J* 1956 *64* 145—150

KATZMAN R L GRAZIANI R KAPLAN and A ESCRIVA Exchange of cerebrospinal fluid potassium with blood and brain *Arch Neurol* (Chic) 1965 *13* 513—524

LEUSEN I and G DEMPESTER, Au sujet de l'action centrale du glucose *Arch int Physiol* 1949 *57* 227—228
MADDOCK S, J E HAWKINS and E HOLMES The inadequacy of substances of the glucose cycle for maintenance of normal cortical potentials during hypoglycemia produced by hepatectomy with abdominal evisceration *Amer J Physiol* 1939 *125* 551—565
MAREN T H Carbonic anhydrase Chemistry, physiology and inhibition *Physiol Rev* 1967 *47* 595—781
MARGOLIS R U and N ALTSZULER Effect of intracisternally administered insulin ^{131}I in normal and vagotomized dogs *Proc Soc exp Biol* (N Y) 1968 *127* 1122—1125
MARK J Y GODIN and P MANDEL Glucose and lactic acid content of the rat brain *J Neurochem* 1968 *15* 141—143
MELLERUP E T and O J RAFAELSEN Brain glycogen after intracisternal insulin injection *J Neurochem* 1969 *16* 777—781
SLOVITER H A and K SAKATA Inactivity of cerebrospinal fluid in regulation of blood glucose concentration *Amer J Physiol* 1963 *204* 153—156
STEWART M A W R SHERMAN, M M KURIEN, G I MOONSAMMY and M WISGERHOF Polyol accumulations in nervous tissue of rats with experimental diabetes and galactosaemia *J Neurochem* 1967 *14* 1057—1066
VUYLSTEKE C A Glycorachie et glycemie *Arch int pharmacodyn* 1949 *78* 456—460
WOODWARD D L D J REED and D M WOODBURY Extracellular space of rat cerebral cortex *Amer J Physiol* 1967 *212* 367—370

Acta physiol scand. 1970. 80. 131—141

From the Department of Physiology, University of Göteborg Sweden

Adrenergic Excitatory Influences on Initiation and Conduction of Electrical Activity in the Rat Portal Vein

By

BENGT LJUNG and LARS STAGE

Received 18 February 1970

Abstract

LJUNG B and L STAGE *Adrenergic excitatory influences on initiation and conduction of electrical activity in the rat portal vein* Acta physiol scand 1970 *80* 131—141

The longitudinal smooth muscle of the rat portal vein shows synchronized spontaneous contractions Both initiation and propagation of this activity are of myogenic nature Under the influence of noradrenaline (NA), or postganglionic nerve stimulation the contraction frequency is known to increase markedly In order to analyse pacemaker function and synchronization of activity in the isolated portal vein both during spontaneous activity and during NA induced excitation a method was designed which allowed simultaneous recording of the mechanical contractions and of the electrical activity from three separate areas in the preparation [illegible] endogenous NA suggest that myogenic propagation plays a most important role in the effectiveness of nervous control of this type of vascular smooth muscle

The longitudinal muscle of the rat portal vein spontaneously exhibits intermittent rhythmic contractions resembling in many respects the vasomotion observed in other sections of the circulatory system Electrophysiologically, this phasic activity of the portal vein has been found to be associated with bursts of spike potentials recorded on crests of slow wave depolarizations (Funaki and Bohr 1964 Axelsson *et al* 1967) Such coordinated rhythmic activity implies that excitation is initiated in a cyclic manner in some part of the tissue, and then propagated to an extensive number of muscle fibres so that a synchronized activation results It has been shown that single muscle cells in the rat portal vein preparation can exhibit gradual depolarization which apparently leads to firing of a burst of action potentials (Funaki and Bohr

1964, Axelsson *et al* 1967) Areas where the cells show this behaviour might thus serve as pacemakers, provided their activity is conducted to other parts of the muscle The contraction frequency of the preparation would then be dependent on the number of active pacemaker areas and the rate of burst formation from each of these The amplitude and duration of this mechanical response, in turn, is determined by the degree of synchronization of the smooth muscle activity (Biamino and Kruckenberg 1969) and by the number and possibly the frequency of the individual spike potentials fired within each burst (Axelsson *et al* 1967) Variations in the effectiveness of excitation contraction coupling may also influence the magnitude of the mechanical response (Johansson *et al* 1967)

Stimulation of the sympathetic vasomotor nerves to the rat portal vein or administration of exogenous noradrenaline (NA), has been found to markedly increase the contraction frequency and to produce tetanus like responses (Johansson *et al* 1967 Johansson and Ljung 1968 Ljung 1969) Such increases in contraction rate may, in principle be caused either by an accelerated frequency of burst activity originating in one pacemaker area or by initiation of activity from 'ectopic' pacemakers

In the present study the influence of exogenous and locally released NA on pacemaker function and propagation of activity in the isolated rat portal vein has been analysed An attempt has been made to elucidate the number of areas emitting waves of burst activity (pacemakers) and the respective frequencies of these during spontaneous basal activity and under the influence of NA Further, the velocity the range and the mode of conduction of the activity have been studied

The method used allowed simultaneous recording of the mechanical activity and of the electrical activity at three different discrete areas of the preparation A similar ique has been used in studies on visceral smooth muscle by *e g* Bortoff (1961)

Methods

Isolated preparations of the portal veins from male rats of the Sprague Dawley strain weighing approximately 500 g were studied in 22 experiments

After sacrifice of the animal by a blow over the neck the portal vein was carefully dissected free from surrounding tissue and cut at both ends A longitudinal strip measuring about 15×4 mm was then prepared It was mounted horizontally usually with the intimal side up in a 30 ml organ bath filled with Krebs solution The mesenteric end (M in Fig 1) was

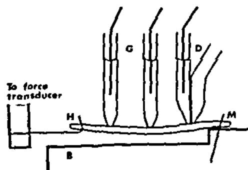
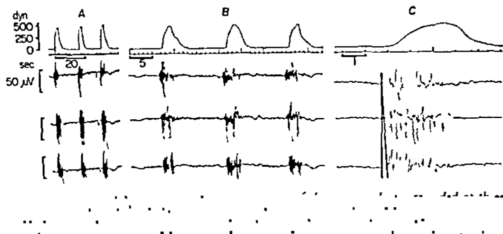


Fig 1 Schematic drawing of experimental set up For explanation see text



attached with six fine needles to a plastic base plate (B) under gentle stretch in the transverse direction The hepatic end (H) was connected, in a similar way, to a force transducer (Grass FT 03), allowing recording of the mechanical activity on a Grass polygraph After a passive stretch of 200—300 dynes had been applied, the strip became freely suspended, at its approximate *in vivo* length, in the Krebs solution, as illustrated in Fig 1

... electrode in the bath ... ace ... to ... the bath by capillary action to make contact with inserted silver wires The electrical signals were amplified and recorded on three channels of the polygraph (Grass preamplifier mod 7 P1A) *via* capacitors (time constant 0 4) The general configuration of the electrograms recorded with this technique was identical whether the tip of the electrode resided in the Krebs solution immediately above the preparation or exerted a gentle pressure upon the suspended muscle It ...

with a syringe operated by a micrometer screw

The modified Krebs solution used had the following composition in mM NaCl 122, KCl 4 73, $CaCl_2$ 2 49, $MgCl_2$ 1 19, $NaHCO_3$ 15 5, KH_2PO_4 1 19, glucose 11 5 and $CaNa_2$-versenate 0 026 It was continuously bubbled with 4 per cent CO_2 in O_2 The temperature was kept at 38° C unless otherwise stated

Results

Spontaneous activity and NA responses at 38° C

The spontaneous activity of the rat portal vein in normal Krebs solution, recorded at three different paper speeds, is shown in Fig 2 A characteristic regular rhythm is seen where the intermittent mechanical contractions are associated with bursts of rapid pontential changes ("spike potentials") in all three leads In this experiment the electrical activity was first observed in the upper recording (see Fig 2 C) and

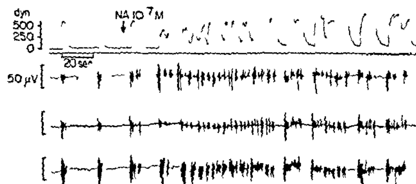


Fig 3 Excitatory response to NA 10^{-7} M Note the marked increase in burst frequency

appeared with increasing delay in the tracings from the second and third electrodes respectively This indicates that bursts of activity were rhythmically initiated in that part of the preparation which was closest to the upper electrode This area thus functions as the pacemaker from which the activity spreads to other parts of the vessel wall The apparent localization of such active pacemaker areas varied between experiments and shifts frequently occurred in the course of the spontaneous activity during an experiment

Recordings like that in Fig 2 C allowed a rough estimation of the conduction velocity by dividing the distance between the electrodes by the corresponding time [illegible]s in the appearance of electrical activity The values were found to vary between 5 and 30 mm/sec

The excitatory responses to moderate concentrations of exogenous NA (10^{-7} M in Fig 3) were characterized by an initial period of frequent bursts of electrical activity which produced an incomplete tetanus As seen in the right part of the recordings of Fig 3 increasing periods of electrical quiescence appeared during continued exposure to NA and the mechanical response then became intermittent with phasic contractions of enhanced duration and amplitude The frequent excitation waves observed under the influence of NA mostly seemed to originate from the same pacemaker area as during the preceding spontaneous activity This indicates that the preparation during NA exposure was driven at a higher rate by the same pacemaker region However propagated activity could often be seen to emanate from other parts of the vessel resulting in variable sequences of excitation and interference phenomena

Greater NA concentrations produced a more or less continuous spike activity in all leads and a tetanic type of maintained tension response The amplitude of the spike potentials diminished with rising NA levels and at 10^{-5} M or higher no spike activity was recorded with the present technique

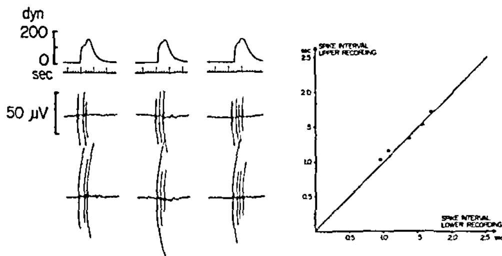


Fig 4 Three consecutive periods of spontaneous electrical and mechanical activity of the rat portal vein at 25° C Note similarities in pattern of spike potential modulation Time between spikes within each burst in lower lead plotted against corresponding values from upper lead in diagram at right Crosses (×) triangles (△) and circles (○) refer to values from left middle and right panels of electrograms respectively Line of identity drawn r = 1 0 Time signal shows marks at 1 and 5 sec intervals

Spontaneous activity and NA responses at 25° C

The experiments presented above gave information about the occurrence and spread of excitation waves as recorded in the form of bursts of spike activity The configuration of these complexes in the different leads hardly permitted any conclusions as to whether they represented a series of conducted action potentials or if they were locally initiated by propagated slow waves of depolarization However at a bath temperature of 25° C the number and frequency of the individual spike potentials were considerably reduced so that the pattern of electrical activity within the burst could be analysed under these experimental conditions Fig 4 illustrates the mechanical activity and the tracings from two of the electrodes (3 mm apart) during spontaneous activity recorded at 25° C Three consecutive spontaneous contractions at a frequency of about one per minute are shown The number and grouping of the spike potentials show a striking resemblance in the two leads The constant time relationship between the electrical events in the lower and upper recordings respectively is demonstrated in the diagram to the right in Fig 4 The intervals between the spikes within each burst in the respective lead were measured and plotted against each other The correlation to the line of identity shows that each individual spike at one recording site had its direct correspondence at the other site indicating that the spikes themselves were propagated under these experimental conditions

When exogenous NA was administered at a bath temperature of 25° C basically the same pattern of contraction ensued as was seen at 38° C However the electrical

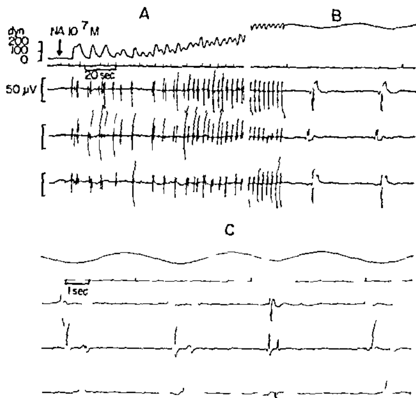


Fig 5 Excitatory response to NA 10^{-7} M at 25° C
A Initial irregular pattern due to interference between activity from multiple pacemaker
B 2 min later Synchronous activity originating from one pacemaker area near the middle rode
C 3 min later than B Waves of activity rhythmically spreading from one end of the preparation (upper lead to the other one and back again Note disturbance in electrical and mechanical regularity due to ectopic excitation recorded in lower lead

activity now consisted of propagated spike potentials of rather low frequency, which facilitated the analysis of the excitation sequences in the vascular muscle during NA induced activity Upon administration of NA (10^{-7} M) tension was elicited by waves of excitation spreading from at least three different pacemaker areas as judged from the pattern of spike activity (Fig 5 A) After 2 min one area apparently located near the middle electrode tended to gain control of the entire preparation which regularly contracted in synchrony at a high rate (Fig 5 B) Fig 5 C shows the activity 3 min later while NA was still present The contraction frequency was somewhat reduced and the sequence of spike potential firing indicated that the preparation was now driven from a pacemaker area located in the hepatic end of the preparation (upper electrogram The excitation waves spread from that end of the muscle strip to the other and evidently back again Such activity patterns which

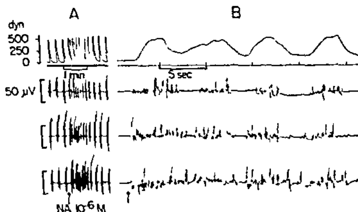


Fig 6 Excitatory effects of local administration of 3 µl of a 10^{-6} M NA solution close to the electrode recording the lower lead Note rapid onset of activity in all three channels and decrease in burst duration with distance from site of NA application

were often seen during periods of NA exposure, but never in the control state, indicates facilitated conduction These experiments seem to establish that NA exerts its excitatory effect both in accelerating pacemaker discharge and possibly also in improving intercellular propagation

Excitatory responses to local NA administration and tyramine

In order to study the conduction of activity when one area of the portal vein was selectively exposed to NA small quantities of this agent were applied topically in 5 experiments *via* one lumen of a double barreled pipette (D in Fig 1) while the electrical activity at this site was recorded in the usual way from the other barrel Electrical activity at two other sites was recorded in the usual manner These experiments were carried out at 38° C In Fig 6 (A and B) 3 µl of a 10^{-6} M NA solution was injected at the time indicated by the arrow, close to the electrode which recorded the lower electrogram The excitatory response was characterized by an almost continuous spike activity at the site of injection and also by considerable increases in the frequency of the bursts in the other two leads (A) The burst duration was least in that part of the vessel which was most distant from the double electrode (B) The mechanical response showed an increased contraction frequency which faded off within 1—2 min The remote excitation observed can be ascribed to propagation of activity from the NA exposed area of the preparation. The rapid onset of activity in all three leads speaks against any significant importance of NA diffusion in producing the response Furthermore the amounts of NA applied were rapidly diluted to subthreshold concentrations in the bath solution

The indirectly acting adrenergic substance tyramine was administered in 5 experiments to illustrate the effects of endogenous NA after its release from the adrenergic nerve terminals The potency of tyramine was 100—1000 times less than that

of exogenous NA The electrical and mechanical excitation in response to 10^{-4} M tyramine in the bath was slower in onset, but resembled very closely in all other respects that to NA illustrated in Fig 3 At 25° C the effect of tyramine was nearly abolished which could indicate that the adrenergic neuronal uptake mechanism was impaired at this temperature

Discussion

The method used in this study was found appropriate to provide information about initiation and propagation of activity in the smooth muscle of the portal vein It was quite easy to handle and did not seem to markedly interfere with the function of the preparation which usually exhibited largely unchanged spontaneous activity and responsiveness for many hours The configuration of volume conducted potentials recorded with relatively large extracellular electrodes of this type, is a function of the geometry and core conductor properties of the tissue (see *e g* Hoffman and Cranefield 1960 Prosser and Bortoff 1968) These properties are as yet not known in detail for this particular smooth muscle and therefore the present results have not been analysed with regard to the shape of the potential changes As to the general pattern of electrical discharge during spontaneous activity and excitatory responses to exogenous NA in the rat portal vein the tracings obtained with the present method closely agree with findings in previous studies where intracellular microelectrodes (Funaki and Bohr 1964 Funaki 1967) or the sucrose gap technique (Johansson *et al* 1967) have been used It therefore seems reasonable to assume that the rapid potential changes recorded during each burst of activity represent compound action potentials discharged in the vicinity of the electrode tip In the present recordings a e of excitation distinguished itself as a rather distinct onset of spike activity since of ible slow waves were filtered away

Earlier studies showed that the automaticity of the portal vein is not abolished by pharmacological nerve blockade (Johansson and Ljung 1967 a b) or by complete degeneration of the sympathetic nerve supply (Johansson *et al* 1969) Both initiation and propagation of the spontaneous activity are thus due to myogenic events The present experiments demonstrate that the smooth muscle cells in different parts of the vessel can act as pacemakers although one area of the preparation presumably that which at the moment has the highest discharge frequency can control the spontaneous activity of the entire muscle layer From such a pace setting region electrical activity spreads along the preparation at a rate of 5—30 mm/sec This velocity is of the same order of magnitude as previously found in rat portal vein by Funaki 1967 in rat aorta (Biamino and Kruckenberg 1969) and in various preparations of visceral smooth muscle of the propagating type (*e g* Burnstock and Prosser 1960) Due to the variable sequences of excitation when NA was added it was not possible to determine with accuracy the influence of NA on conduction velocity under these experimental conditions

In principle the wave of excitation emanating from the pacemaker area might be

represented either by a series of conducted action potentials or alternatively by an electrotonically propagated 'slow wave' which would act as a local generator potential to initiate action potentials in the subsequent parts of the muscle This question concerning the mechanism of conduction in the portal vein could not, however, be settled by analysis of the recordings obtained at 38° C On the other hand the observations of consistent spike intervals at different recording sites obtained at the reduced level of activity at 25° C (Fig 4) suggest that the spike itself can be conducted over considerable distances in the portal vein as in other types of smooth muscle (Burnstock and Prosser 1960 Tomita 1967) This would indicate that the pacemaker area rhythmically sends out trains of propagated action potentials, the 'bursts', which then become modified during their course along the preparation possibly as a result of failing conduction and sometimes because of interference from activity originating in 'ectopic" pacemakers The modulation of the burst with increasing distances of conduction is clearly illustrated in Fig 6

During exposure to moderate NA concentrations the frequency of bursts greatly increased as previously reported (Johansson *et al* 1967) Both when the effects were studied at 38° C and at 25° C it was evident that NA accelerated the discharge rate of the original pacemaker, responsible of the spontaneous activity during the preceding control period, so that the preparation became synchronously activated from this area by bursts at higher frequencies In addition 'ectopic' pacemakers could now emit propagated waves of excitation which led to irregular patterns of electrical and mechanical activity

The smooth muscle showed a marked tendency to discharge intermittently even under the excitatory influence of low and moderate concentrations of NA During continued NA exposure gradually longer periods of electrical inactivity appeared the duration of which approached that of the silent periods during the control state These phenomena, which thus regulate both the duration and incidence of bursts seem to reflect inherent cyclic changes in the excitability of the smooth muscle

The rhythmic behaviour of the preparation gradually disappeared when NA was administered in high concentrations (10^{-5} M or more) As discussed by Johansson *et al* (1967), who recorded similar results with the sucrose gap method this may be due to a maintained depolarization leading to a contracture, or it may represent an asynchronous spike activity of the individual cells with a tetanic type of contractile response A differentiation between these alternatives was not possible with either of these extracellular recording techniques

It was reported in previous studies that exogenous NA and sympathetic nerve stimulation gave rise to mechanical responses of similar patterns in the portal vein (Johansson and Ljung 1967 a 1968 Ljung 1969) Holman *et al* (1968) emphasized this similarity also in their electrophysiological study on rabbit portal vein with the sucrose gap technique Attempts were made to use the present method for recording electrical phenomena during neurogenic responses of the vascular preparation elicited by stimulation of the dissected postganglionic nerve supply This was however complicated by considerable stimulus artifacts Instead tyramine,

ministered to allow recordings of the effects of endogenous NA Tyramine is considered to be actively taken up into the varicosities of the adrenergic terminal fibres causing liberation of NA (For ref see Trendelenburg 1963) The patterns of the electrical and mechanical responses to tyramine were in every respect similar to those obtained by exogenous NA As the portal vein, like most vascular smooth muscle is supplied with a sparse adrenergic innervation which does not penetrate into the muscle bundles of the media (Johansson *et al* 1969), only a limited portion of the muscle cells seems to be directly affected by the released transmitter However, a considerable spread of excitation was seen to occur when a focal adrenergic stimulation was applied in the present experiments (Fig 6) Evidence was obtained in a previous study (Johansson and Ljung 1968) that a similar mechanism can operate during nerve activity so that neurogenically induced excitation of a limited portion of the muscle may influence the activity in the entire portal vein due to the propagating ability of this vascular smooth muscle

We are grateful to Dr Borje Johansson for much help and advice and to Mrs Eva Bengtsson Miss Christine Lind and Mrs Siv Samuelsson for able technical assistance
[illegible] Un[illegible]ers[illegible]y of [illegible]

Nor Exadrin® conc was generously supplied by AB Astra

References

Axelsson J B Wahlstrom B Johansson and O Jonsson Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein *Circulat Res* 1967 *21* 609—618

Bjamino G and P Kruckenberg Synchronization and conduction of excitation in the rat aorta *Amer J Physiol* 1969 *217* 376—382

Bortoff A Slow potential variations of small intestine *Amer J Physiol* 1961 *201* 203—208

Burnstock G and C L Prosser Conduction in smooth muscles comparative electrical properties *Amer J Physiol* 1960 *199* 553—559

Funaki S Electrical and mechanical activity of isolated smooth muscle from the portal vein of the rat In Symposium on electrical activity and innervation of blood vessels Cambridge 1966 *Bibl anat* Basel 1967 *8* 5—10

Funaki S and D F Bohr Electrical and mechanical activity of isolated vascular smooth muscle of the rat *Nature* Lond) 1964 *203* 192—194

Hoffman B F and P F Cranefield *Electrophysiology of the heart* McGraw Hill Book Co Inc 1960 pp 323

Holman M E C B Kasby M B Suthers and J A F Wilson Some properties of the smooth muscle of rabbit portal vein *J Physiol* (Lond) 1968 *196* 111—132

Johansson B O Jonsson J Axelsson and B Wahlstrom Electrical and mechanical characteristics of vascular smooth muscle response to norepinephrine and isoproterenol *Circulat Res* 1967 *21* 619—633

Johansson B and B Ljung Sympathetic control of rhythmically active vascular smooth muscle as studied by a nerve muscle preparation of portal vein *Acta physiol scand* 1967 a *70* 299—311

Johansson B and B Ljung Spread of excitation in the smooth muscle of the rat portal vein *Acta physiol scand* 1967 b *70* 312—322

Johansson B and B Ljung Role of myogenic propagation in vascular smooth muscle response to vasomotor nerve stimulation *Acta physiol scand* 1968 *73* 501 510

JOHANSSON, B., B. LJUNG, T. MALMFORS and L. OLSON, Denervation supersensitivity in the sparsely innervated smooth muscle of the portal vein *Acta physiol scand* 1969 77 Suppl. 330 58

LJUNG, B., Local transmitter concentrations in vascular smooth muscle during vasoconstrictor nerve activity *Acta physiol scand* 1969 77 212—223

smooth muscle fibres *Amer*

muscle under in vitro condi- 5—2050

nea pig taenia coli *J Physiol*

npathomimetic amines *Phar-*

Acta physiol. scand. 1970. 80. 142—144

From the Brain Research Laboratory, E-blocket, the University Hospital, and from the Department of Anesthesia, University of Lund, Sweden

The Effect of Anesthetics on the Tissue Lactate, Pyruvate, Phosphocreatine, ATP and AMP Concentrations, and on Intracellular pH' in the Rat Brain

By

L. NILSSON and B. K. SIESJÖ

It has been reported that different anesthetics (barbiturates, ether and chloroform) increase the phosphocreatine and decrease the lactate concentrations of brain tissue (Stone 1938, Mayman *et al.* 1964, Gatfield *et al.* 1966), also that these effects are related to the depth of the anesthesia (Goldberg *et al.* 1966). These results thus indicate that anesthetics in general decrease the utilization of high energy phosphates and that anesthesia represents a high energy state (*cf.* McIlwain 1966). However, the results are sometimes difficult to interprete since the freezing and extraction techniques used undoubtedly have allowed some autolytic changes to occur [be]fore analyses, and since the physiological states of the animals have been insuf[ficient]ly controlled.

We have studied the effects of barbiturates and of various other anesthetics on the tissue concentrations of lactate, pyruvate, ATP, AMP and phosphocreatine, and on intracellular pH' in the rat brain, utilizing fixation and extraction techniques which minimize autolytic changes (see Kaasik *et al.* 1970). In order to achieve a comparable physiological state in the groups, all animals were immobilized and artificially ventilated so as to give an arterial CO_2 tension of 35—40 mm Hg and 30 % oxygen was administered in the insufflated gas mixture throughout the experiments. In all animals except those given anesthetic doses of phenobarbital, anesthesia was induced with divinyl ether. Three groups of animals were given phenobarbital i.p. in doses of 50, 150 and 250 mg/kg, respectively. In the other 3 groups the depth of anesthesia was varied by giving either 0.013 mg/kg of fentanyl s.c., 70 % nitrous oxide or 7 % ether. In the fentanyl group the drug was given during anesthesia with nitrous oxide, which was withdrawn when the drug was given. In all groups of rats the brain was frozen *in situ* after a 30—50 min anesthetic period. Techniques and procedures used have been described previously (Kaasik *et al.* 1970), and additional details will appear in a forthcoming publication. All metabolite concentrations were measured enzymatically, while pH' was calculated from the tissue bicarbonate con-

TABLE Tissue concentrations of lactate, pyruvate, ATP, ADP, AMP and phosphocreatine, as well as the calculated intracellular pH_i in the brains of rats, anesthetized with various anesthetics Means ± S E

Exp group	PCr	ATP	AMP	La	Py	La/Py	pH_i
	mmoles/kg	mmoles/kg	mmoles/kg	mmoles/kg	mmoles/kg		
Fentanyl n = 6	4 72 ±0 16	2 81 ±0 04	0 02 ±0 00	1 33 ±0 07	0 083 ±0 001	16 0 ±0 4	7 102 ±0 006
Nitrous oxide n = 7	5 04 ±0 06	2 80 ±0 04	0 02 ±0 01	1 56 ±0 07	0 098 ±0 005	16 1 ±0 6	7 089 ±0 017
Diethylether n = 14	5 23 ±0 05	2 85 ±0 03	0 02 ±0 00	1 51 ±0 08	0 096 ±0 005	16 0 ±0 6	7 102 ±0 010
Phenobarb 50 mg/kg n = 6	5 21 ±0 07	2 84 ±0 07	0 01 ±0 00	1 11 ±0 07	0 083 ±0 005	13 4 ±0 4	7 125 ±0 007
Phenobarb 150 mg/kg n = 6	5 38 ±0 15	2 82 ±0 06	0 01 ±0 00	0 97 ±0 12	0 078 ±0 007	12 2 ±0 7	7 143 ±0 010
Phenobarb 250 mg/kg n = 8	5 30 ±0 05	2 81 ±0 02	0 02 ±0 00	0 88 ±0 05	0 078 ±0 003	11 5 ±0 6	7 173 ±0 008

centration after correction for the bicarbonate contained in a 3 % blood and a 12 % extracellular fluid volume

The results of the experiments are given in the table The mean arterial CO_2 tensions of the groups varied between 36 7 and 39 6 mm Hg The table shows that the type or depth of anesthesia had no significant effects on the tissue concentrations of ATP or AMP and a very small if any, effect on the phosphocreatine concentrations Phenobarbital was associated with decreases in the lactate concentration, and in the lactate/pyruvate ratio and with an increase in pH_i', and these effects *appeared to be dose dependent*

The present results indicate that anesthesia has a negligible influence on labile phosphates in the brain and that previous results showing a marked effect of anesthetics on *e g* the phosphocreatine concentration have been significantly influenced by autolytic changes occurring during the freezing Our results also indicate that the lowering of the tissue lactate concentration is specific for barbiturates and that such drugs also lead to decreases in the lactate/pyruvate ratio, and to increases in the pH_i' The general conclusions have been found to apply also to other volatile anesthetics (halothane cyclopropane) and to other barbiturates (thiopentone, amobarbital)

Supported by the Swedish Medical Research Council (Project B69 14X 263 05), by the Swedish Bank Tercentary Fund and by the NIH (Grant No 1Rol NB 7838 01)

References

GATFIELD, P. D., O. H. LOWRY, D. W. SCHULZ and J. V. PASSONNEAU, Regional energy reserves in mouse brain and changes with ischemia and anesthesia. *J. Neurochem.* 1966. 13: 185—195.

[illegible]

1966. p. 43 and 338—345.

STONE, W. E., The effects of anesthetics and of convulsants on the lactic acid content of the brain. *Biochem. J.* 1938. 32: 1908—1918.

Acta physiol scand 1970 80 145—148

From the Department of Pediatrics and Pediatric Anesthesiology S t Görans Children s Hospital Karolinska Institutet Stockholm Sweden

Effect of Hypotonic Mannitol and Saline Load on Diluting Capacity in Man

By

A Aperia O Broberger and H Feychting

Received 3 December 1969

Abstract

Aperia A O Broberger and H Feychting *Effect of hypotonic mannitol and saline load on diluting capacity in man* Acta physiol scand 1970 *80* 145—148

The effect of hypotc
has been examined
free water formatic
demonstrated that th
by progressive incre
demonstrated between distal tubular Na reabsorption and urinary Na concentration This was interpreted to mean that the distal tubular Na reabsorption was independent of the transtubular Na gradient

It is generally agreed that the dilution of urine is mainly a function of active Na+ reabsorption in the water impermeable thin ascending limb and early distal tubule Evidence for this assumption is the finding that differences in Na concentration accounts for at least 91 % of the differences in osmolality between adjacent descending and ascending limbs of the loop of Henle (Berliner and Bennett 1967) The rate of solute free water production can therefore be used as an index of distal tubular Na reabsorption Several studies mainly on dogs have demonstrated that when the Na supply to the distal tubule is increased by mannitol or saline diuresis there is at first a steep rise in free water production parallelling increments in osmolar clearance and urine flow (Earley *et al* 1961 Eknoyan *et al* 1967 Goldstein *et al* 1961 Rector *et al* 1968 Stein *et al* 1967) In some studies however the free water production will finally tend to stabilize despite progressively increasing delivery of filtrate and Na Thus it might be proposed that the distal tubule exhibits the same type of supply dependent kinetics that has been described for the proximal tubule (Lewy and Windhager 1968 Ullrich *et al* 1963)

The present study is an attempt to evaluate the distal tubular Na reabsorption in man For this purpose the Na delivery to the distal tubules was varied over a wide range by the infusion of hypotonic mannitol and of hypotonic saline

Material and methods

This study was carried out in 14 young healthy male volunteers without histories of previous nephropathies

On the day of the study the patients were given a standard breakfast meal Thereafter no food was allowed until the end of the study Water diuresis was induced by oral intake of water in amounts corresponding to 2 % of the body weight initially and thereafter 0 5 % of the body weight every 30 min For saline diuresis (9 studies) a solution of hypotonic NaCl was infused i v at a rate slightly exceeding the diuresis The concentration of NaCl was gradually increased during the course of the study from 0 36 to 0 72 % For mannitol diuresis (5 studies) a 3 % solution of mannitol in water was given i v at a rate slightly exceeding the diuresis

For infusions and blood sampling needles were placed into 2 superficial veins For the i v infusion of the hypotonic mannitol or saline solution the inferior vena cava was catheterized with Seldinger technique The bladder was catheterized with a double lumen catheter allowing continuous suction

The blood pressure was recorded repeatedly with cuff and mercury manometer

Standard clearance techniques were used The glomerular filtration rate was determined with the clearance of inulin (Laevasar Gesellschaft Gallva AB) For this purpose inulin was 0 001 g/kg b w /min Blood samples were withdrawn in the middle of each urine collection given in an intravenous prime dose of 50 mg/kg b w followed by the continuous infusion of period

Urine and serum samples were analyzed with regard to concentration of inulin Na^+ and osmolality Inulin was determined according to the method of Heyrovsky (Heyrovsky 1956) Na^+ was determined in an Eppendorf flame photometer Osmolality was determined cryoscopically with a Knauer micro osmometer

Results

The arterial blood pressure and the glomerular filtration rate tended to remain stable throughout each experiment Table I demonstrates the average values for the glomerular filtration rate towards the end of saline load mannitol load and in water diuresis alone No significant difference could be found between the glomerular filtration rate during the three stages

The following analysis of data are built on the assumption that the free water formation (C_{H_2O}) is determined by distal tubular Na reabsorption Thus the sum of free water and Na clearances ($C_{H_2O}+C_{Na}$) should be an index of distal tubular Na^+ delivery Fig 1 demonstrates the relationship between distal tubular Na reabsorption (C_{H_2O}) and distal tubular Na delivery ($C_{H_2O}+C_{Na}$) Both parameters have been related to the glomerular filtration rate All data from mannitol and saline studies are included Initially the free water production appears to rise steeply with increments in distal tubular Na delivery The relationship between distal tubular Na^+ reabsorption and distal tubular Na delivery does however reach a maximum when the sum of the free water and Na clearances is around 16 ml/100 ml GFR At

TABLE I The glomerular filtration rate during various diuretic stages The values are expressed in ml/min 1 73 m² body surface The calculations include the last two recordings from each individual during mannitol and during saline infusion

	mean	S D	n
water diuresis	109	13	13
water diuresis with superimposed hypotonic mannitol load	106	10	18
water diuresis with superimposed hypotonic saline load	110	14	8

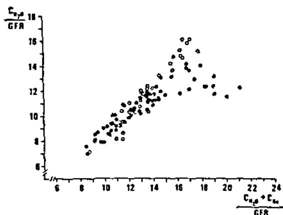


Fig 1 The relationship between free water production (C_{H_2O}) and distal tubular Na delivery ($C_{H_2O}+C_{Na}$) Both parameters are related to the glomerular filtration rate The open circles represent values obtained during mannitol experiments The dots represent values obtained during saline experiments

further increments in distal tubular Na^+ delivery the free water production appears to decline

In Fig 2 the free water clearance (C_{H_2O}) has been plotted against the urinary Na^+ concentration The urinary Na^+ concentration ranges between 6 meq/l and 50 meq/l The values above 25 meq/l were all obtained during saline diuresis There is no apparent relationship between urinary Na^+ concentration and free water production Thus increased urinary Na^+ concentration does not enhance the distal tubular Na^+ reabsorption

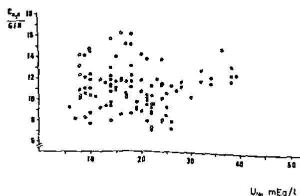


Fig 2 The relationship between free water production (C_{H_2O}) and urinary Na^+ concentration The free water production is related to the glomerular filtration rate The open circles represent values obtained during mannitol experiments The dots represent values obtained during saline experiments

Discussion

A previous study in hydrated man where distal tubular Na^+ supply was increased only by means of mannitol infusion suggested that the free water formation was limited (Aperia *et al* 1969) Mannitol will however, dilute the tubular fluid and thus lower the Na^+ concentration (Ullrich *et al* 1963) It was therefore postulated that any further increase of the distal tubular Na reabsorption was restricted by the transtubular Na gradient and that the reabsorptive capacity might be further increased by a reduction of the gradient The net flux of water and ions in the collecting ducts is negligable during water diuresis Thus the Na concentration in u

should be representative for the concentration in the distal tubular fluid No positive relationship was found between distal tubular Na^+ reabsorption and urine Na^+ concentration Thus the proposed mechanism for the limiting effect of mannitol on distal tubular reabsorptive capacity was proved to be wrong In fact the present findings strongly suggest that the distal tubular Na^+ reabsorption is independent of *the transtubular Na^+ gradient*

If distal tubular Na reabsorption is related to the total rate of distal Na^+ delivery a positive relationship could to a certain extent be demonstrated between the two factors Increases in the sum of free water and Na^+ clearances from 8 to 16 ml/100 ml GFR resulted in a steep rise in the free water production At further increments in distal tubular Na^+ delivery the free water production started to decline however Thus the distal tubular Na reabsorption appears to be at first enhanced and later suppressed by increasing distal tubular Na^+ delivery The present study does however not further reveal the mechanism of the supply dependent kinetics just described The importance of physical factors for distal tubular Na reabsorption is unknown Those authors that have described a natriuretic hormone have not attributed any distal effects to it (Rector *et al* 1968)

Supported by Swedish Medical Research Council Grant B67—19X 2049 01A
The authors are indebted to Mrs Karin Egeld and miss Guye Svensson for expert technical assistance

References

Aperia A O Broberger N O Ericsson and H Feychting Renal function in man during transition from hydropenia to water diuresis with superimposed mannitol load *Acta physiol scand* 1969 *77* 429 438

Berliner R W and C M Bennett Concentration of urine in the mammalian kidney *Amer J Med* 1967 *42* 777 789

Earley L E M Kahn and J Orloff The effects of infusions of chlorothiazide on urinary dilution and concentration in the dog *J clin Invest* 1961 *40* 857 866

Eknoyan G W N Suki F C Rector and D W Seldin Functional characteristics of the diluting segment of the dog nephron and the effect of extracellular volume expansion on its reabsorptive capacity *J clin Invest* 1967 *46* 1178 1188

Goldstein M H M F Levitt A D Hauser and D Polimeros Effect of meralluride on solute and water excretion in hydrated man Comments on site of action *J clin Invest* 1961 *40* 731 742

Heyrovsky A A new method for the determination of inulin in plasma and urine *Clin chim Acta* 1956 *1* 470 474

Lewy J E and E E Windhager Peritubular control of proximal tubular fluid reabsorption in the rat kidney *Amer J Physiol* 1968 *214* 943 954

Rector F C G van Giesen F Kiil and D W Seldin Influence of expansion of extracellular volume on tubular reabsorption of sodium independent of changes in glomerular filtration rate and aldosterone activity *J clin Invest* 1964 *43* 341—348

Rector F C M Martinez Maldonado N A Kurtzman J C Sellman F Oerther and D W Seldin Demonstration of a hormonal inhibitor of proximal tubular reabsorption during expansion of extracellular volume with isotonic saline *J clin Invest* 1968 *47* 761—773

Stein, R M R. G Abramson T Kahn and M F Levitt Effects of hypotonic saline loading in hydrated dog evidence for a saline induced limit on distal tubular sodium transport *J clin Invest* 1967 *46* 1205 1214

Ullrich, K J, B Schmidt Nielsen S O'Dell G Pehling C W Gottschalk W E Lassiter and M Mylle Micropuncture study of composition of proximal and distal tubular fluid in rat kidney *Amer J Physiol* 1963 *204* 527 531

Acta physiol. scand 1970 80 149—159

From the Department of Physiology, Karolinska Institutet Stockholm Sweden

Mechanisms Involved in the Cat's Blink Reflex

By

CHR LINDQUIST and A MÅRTENSSON

Received 13 February 1970

Abstract

LINDQUIST, CHR and A MÅRTENSSON *Mechanisms involved in the cat's blink reflex* Acta physiol scand 1970 *80* 149—159

fibers from muscle spindles or other types of proprioceptors in the facial and trigeminal nerves A twitch contraction of the orbicularis oculi elicits a high amplitude reflex response in the muscle through activation of trigeminal exteroceptive afferents the possibility is considered that such afferents may serve a proprioceptive function in monitoring the degree of contra tion of the facial muscles

In investigations of reflexes elicited in human facial muscles Kugelberg (1952 *cf* also Ekbom *et al* 1952) demonstrated that the reflex responses to mechanical stimulation of the face are composed of two successive discharges Studies of the blink reflex revealed that the elicitation of a double response in the orbicularis oculi depends on what area of the face is stimulated, thus a tap around the eye gives rise to both reflex components whereas only a long latency response results on tapping over more remote areas In experiments with electrical stimulation the trigeminal nerve was found to be the afferent link in both these reflex components More recent investigations of facial reflexes in man indicate that also afferent fibers in the facial nerve may mediate both the primary (Rushworth 1962) and the secondary response (Gandiglio and Fra 1967)

In his analysis of the functional properties of the two reflex responses in the human orbicularis oculi Kugelberg (1952) could identify the secondary response as a polysynaptic reflex of exteroceptive origin the early response which was most readily evoked by tapping on the skin overlying the muscle was composed of a w

chronized volley, and by comparing its latency with that of the stretch reflex in the masseter the conclusion was drawn that it was a myotatic reflex This suggests that the facial muscles should be served by a conventional proprioceptive mechanism involving muscle spindles Histological studies of the human facial muscles in the search for muscle spindles have however yielded contradictory results (Baum 1900 Kadanoff 1956), and a closer analysis of these reflexes in animal experiments has been considered pertinent

Of special interest is that also in the cat two successive reflex responses are set up in the facial muscles on afferent stimulation of branches of the trigeminal nerve (Tokunaga *et al* 1958) By observing effects of lesions of the brain stem and subcortical structures it could be established that the two reflex components are mediated through different central pathways and that the early response is transmitted through a simple reflex arc possibly containing two synapses The central relay time of the early response has however not been determined nor has a closer physiological analysis been made to find out whether the facial muscles in the cat may contain spindles Histological studies have not provided any conclusive evidence Thus in a preliminary report Bowden *et al* (1960) described spindles in the cat's facial muscles whereas Bruesch (1944) in degeneration studies of the cat's facial nerve, did not find any type of organized sensory nerve endings in the muscles, the facial nerve was found to contain only a small number of afferent fibers of fine caliber originating in free nerve endings located between the muscle fibers

In the present paper an account will be given of an analysis of the cat's facial reflexes performed both in order to study some mechanisms involved in the blink reflex and to find out whether a proprioceptive system including muscle spindles may serve the facial muscles The first section of the paper will present results from experiments of the type previously carried out in human investigations using facial tap stimulation In the second section it will be shown that both the early and late reflex components are elicited by exteroceptive stimuli and transmitted via trigeminal afferents through multisynaptic reflex pathways Finally a study of facial muscle reflexes evoked by stimulation of high threshold afferent fibers in the facial nerve will be reported as well as some negative results from attempts to record activity from proprioceptive afferents in filaments from the trigeminal and facial nerves

Methods

Tracheotomized cats weighing 2 5 to 3 5 kg were used for the experiments Anesthesia was induced by ether and maintained by injections of chloralose in doses of 50—70 mg/kg bw administered through a catheter in the femoral vein The cat was kept warm with an infrared heating lamp

Tap stimulation was delivered by means of a mechanical stimulator with a high sensitivity capacitance meter Haapanen 1962 recording the movements of the stimulus probe (for details see Nilsson 1969) The moment when contact was made between probe and skin was not recorded and hence the amplitude of the skin displacement and the latency of the resulting reflex responses could not be exactly determined By invariably placing the probe in the immediate vicinity of the skin these values could however be estimated with an exactness sufficient for the purpose of the present experiments

A Grass S 4 stimulator connected to bipolar chlorided silver wire electrodes delivering square

wave stimuli of 0 1 msec duration were used for stimulation of the Gasserian ganglion as well as of trigeminal and facial nerve branches Steel needles insulated to the tip were used for [illegible]

and a Grass P 6 amplifier

Results

Mechanical stimulation

An initial series of investigations was performed using an experimental procedure similar to that employed by Kugelberg (1952) in his studies on man Thus, mechanical stimulation was applied by tapping on different areas of the face and recordings made from the orbicularis oris the quadratus labii superior and the orbicularis oculi muscles In the orbicularis oris no responses whatever were set up on stimulation of any parts of the face In the quadratus labii superior—as in the human perioral musculature (Ekbom *et al* 1952)—two successive reflex responses were obtained by tapping on the skin overlying the muscle Since, however, similar responses could most readily be elicited in the orbicularis oculi, this muscle was selected for a more detailed analysis

A typical recording from the orbicularis oculi on stimulation of the skin immediately over the muscle is shown in Fig 1 *A* The response is composed of two successive reflex discharges of a duration of 8 and 10 msec respectively In successive stimulations of equal amplitude the latency variations of the early response were but a few msec, *i e* 9—12 msec whereas the latencies of the late response varied over a wider range *i e* 15—25 msec As in human experiments a stimulus strength around threshold might sometimes give rise only to the early response, sometimes only to the late response, but just as often both responses were set up The threshold for mechanical stimulation was thus the same for both reflexes

As shown in Fig 1 *B* and *C*, two successive reflex responses were also elicited by tapping on areas more remote from the muscle *e g* the tip of the nose (*B*) or the frontal region (*C*), but the amplitudes of these responses are lower and their latency longer even though the stimulus amplitude is the same as in *A* As in human experi

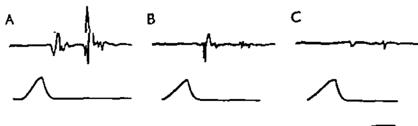


Fig 1 Two successive reflex discharges elicited in the orbicularis oculi (*upper beam* in response to tap stimuli of equal amplitude applied to *A* the skin over the muscle *B* the tip of the nose and *C* the frontal region *Lower beam* indicates movement of stimulus probe Time bar 10 msec

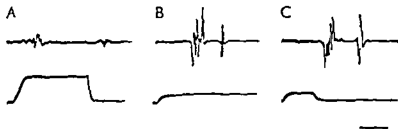


Fig 2 Double reflex discharges elicited in the orbicularis oculi (*upper beam*) in response to tap stimuli of longer duration than those in Fig 1 (*cf lower beams*) Full description in text Time bar 10 msec

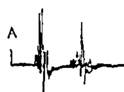
ments it was a general observation that reflex responses evoked by tapping on areas at some distance from the eye were weaker than those obtained by tapping on the skin in the vicinity of the muscle However whereas in man the long latency component of the response was most commonly obtained from more remote sites often only the early reflex component was set up in the cat on such tapping (Kugelberg 1952 *cf* also Discussion)

Fig 2 *A*—*C* shows reflexes set up in the orbicularis oculi in response to tap stimuli of longer duration than those delivered in Fig 1 In *A* the early response is followed by a second discharge which is not elicited until after the probe has lost contact with the skin By comparison with recordings from peripheral filaments of the trigeminal nerve it could be established that this secondary response was caused by an off volley evoked in the afferent fibers on withdrawal of the probe from the skin The recordings in *B* and *C* are from another experiment the stimulus site and amplitude are the same but in *B* the stimulus duration is 40 msec as against 15 msec in *C* Since the late response in *B* is initiated before the probe has lost contact with the skin it cannot be due exclusively to off-effects However the amplitude of the second response is higher when the stimulus duration is briefer (*C*) and consequently such effects may contribute to the amplitude of this response

Owing to the close mechanical interdependence of different facial structures a tap applied to the intact skin is apt to spread to underlying tissues In some of the experiments the skin over the orbicularis oculi was dissected free so that separate stimuli could be applied to skin and muscle Tapping directly on the muscle belly gave no reflex responses whatever Provided that no proprioceptive fibers were cut by the dissection this finding indicates that no intramuscular mechanoreceptors should be involved In response to tapping on the skin flap the characteristic two reflex components were elicited as illustrated in Fig 3 A It is thus evident that exteroceptive fibers contribute to the initiation of both components

Electrical stimulation of trigeminal afferent nerve fibers

In another series of experiments electrical stimulation was applied to trigeminal branches supplying the skin over the orbicularis oculi As appears from Fig 3 *B* af



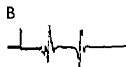
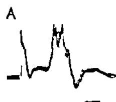


Fig 3



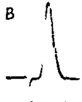


Fig 4

Fig 3 Comparison of double reflex discharges elicited in the orbicularis oculi in [illegible] *A*, tapping on a skin flap (five superimposed sweeps), *B* electrical stimulation of the [illegible] nerve See text Time bar 10 msec

Fig 4 Determination of central relay time of early reflex response by recording from the nerve to the orbicularis oculi *A* reflex discharge elicited by stimulation of the Gasserian ganglion *B* direct response to stimulation of the facial nerve at its exit from the brain stem See text Time bars 2 msec

ferent stimulation of the frontal nerve of a strength required to activate coarse sensory fibers results in a double reflex response similar to that elicited by peripheral stimulation The latency of the early response is 7 msec that of the late response 2[illegible] msec Similar reflex responses were obtained on afferent stimulation of the infraorbital nerve Also on electrical stimulation the thresholds of the two reflex components were similar, and afferent fibers of equal caliber should thus be involved in both reflexes (*cf* Tokunaga *et al* 1958)

Fig 4 illustrates an experiment designed to determine the central relay time of reflexes produced by stimulation of afferent fibers of the trigeminal nerve In *A* an early reflex response, elicited by stimulation of the Gasserian ganglion is recorded in the nerve to the orbicularis oculi after a delay of 4 0 msec In *B* the facial nerve is stimulated at the level of its exit from the brain stem and the efferent volley is recorded in the muscle nerve at the same site as the reflex The efferent conduction time proved to be 0 5 msec and the central relay time is consequently about 3 5 msec The reflex should thus have been mediated through a [illegible] reflex arc According to Fig 3 the difference in latency between the first and second reflex components is 14 msec and the central relay time of the late response is thus 17 5 msec in this case

Electrical stimulation of the facial nerve

As mentioned in the introduction data obtained in human studies suggest that both the early and late reflex components might in part be transmitted through afferent fibers of the facial nerve (Rushworth 1962 Gandiglio and Fra 1967) In order to establish whether afferent fibers in the cat's facial nerve are involved in the elicitation of the reflex responses a series of experiments were performed in which electrical stimulation was applied alternately to each of the three main branches [illegible] were made from the central part of the branch stimulated as well as from [illegible]

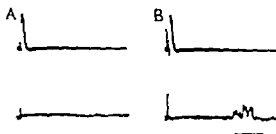


Fig 5 Testing for reflex discharges in the nerve to the orbicularis oculi (*lower beams*) and in the stimulated ventral branch of the facial nerve (*upper beams*) *A* stimulus strength adjusted so as to give maximal direct response in the nerve stimulated *B* sixfold increase in stimulus strength yields reflex discharge Time bar 10 msec

the two other branches Out of the different combinations thus tested only one ει afferent stimulation of the ventral branch which supplies the perioral musculature gave rise to a reflex discharge, which was recorded in the branch to the orbicularis oculi Typical recordings from experiments of this type are shown in Fig 5 *A* and *B* In *A*, the stimulus applied to the central part of the ventral branch in a curarized animal is strong enough to evoke a maximal direct response of the coarse nerve fibers (upper beam), but no reflex response results in this branch nor in the branch to the orbicularis oculi (lower beam) In *B* in which the stimulus strength is six times that in *A*, a low amplitude reflex response is set up in the nerve to the orbicularis oculi after a delay of 25 msec

In another type of experiments performed to find out whether afferent fibers in the facial nerve might be involved in the reflex activity of the facial muscles electrical stimuli applied to the central parts of cut facial nerve branches were followed by electrical or mechanical stimulation of the skin on the face or by electrical stimula n of peripheral branches of the trigeminal nerve Fig 6 *A*—*C* illustrates typical ngs in an experiment in which the conditioning stimulus in this case an electrical nulus applied to the ventral branch of the facial nerve is followed by intradermal test stimuli In the figure non-conditioned reflex responses in the orbicularis oculi are seen at the left hand side and the effects on these reflexes produced by conditioning stimuli at various intervals are shown at the right hand side In *A* the condi

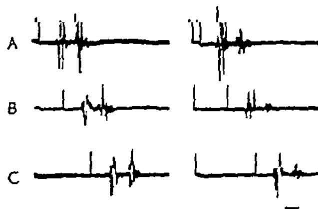
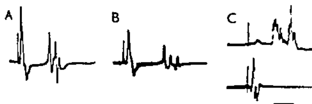


Fig 6 Effect of conditioning stimuli applied to high threshold afferent fibers of the facial nerve upon reflexes elicited in the orbicularis oculi by intradermal stimulation of the facial skin See text Time bar 10 msec

Fig 7 *A* and *B* Direct muscle responses and reflex responses in the orbicularis oculi to stimulation of the intact muscle nerve at maximal respectively submaximal stimulus strength *C* *upper beam* reflex discharge in the central part of the cut nerve to the orbicularis oculi in response to stimulation of its peripheral part *Lower beam* direct discharge in the muscle in response to the peripheral stimulus Time bar 10 msec



tioning test interval is below 10 msec, and there is then a typical increase in amplitude of the early response and a marked depression of the late response With an interval of about 20 msec, as in *B* the conditioning stimulus does not have any distinct effect on the early response but the late reflex component is clearly inhibited When the interval is about 40 msec (*C*), both components are depressed This inhibitory effect could be observed at stimulus intervals up to 200 msec but was most pronounced at intervals below 80 msec Similar results were obtained regardless of whether the reflex responses had been evoked by electrical or mechanical stimulation of the facial skin or by electrical stimulation of the trigeminal nerve Also after conditioning stimuli applied to other facial branches similar effects were obtained However, the test reflexes were not influenced until the strength of the conditioning stimuli was increased to 5—6 times threshold for the motor fibers of the facial nerve Thus, no evidence was obtained that the facial nerve branches should contain any low threshold afferents The experiments demonstrate however that afferent activity in high threshold afferent fibers of the facial nerve may have both excitatory and inhibitory effects on the reflex activity of the facial motoneurons

Fig 7 *A* and *B* shows recordings from the orbicularis oculi in response to stimulation of the intact facial nerve Besides the maximal direct muscle response also a high amplitude reflex discharge is set up 15 msec later As seen in *B* also a stimulus submaximal for the direct response results in a reflex discharge in the muscle The upper trace in *C* shows that a reflex can also be recorded in the proximal part of the cut muscle nerve on stimulation of the peripheral part of the nerve These responses could be abolished by curarization as well as by section of the ipsilateral trigeminal root They should thus be set up by an afferent volley in the trigeminal nerve elicited by activation of exteroceptors responding to muscle contraction Further proof for this concept was obtained in experiments showing that a twitch contraction of facial muscles elicits a high amplitude afferent volley in trigeminal skin afferents (cf Lindquist and Mårtensson 1969 a) These findings will be commented on below in connection with the discussion of the origin of the secondary responses to tapping

Afferent recording from trigeminal and facial nerves

In still another series of experiments recordings were made from peripheral filaments of the facial and trigeminal nerves in an attempt to find out whether or not muscle spindles or mechanoreceptors of other types might occur in the facial muscles

In some of these experiments the skin over the muscles was left intact, in others the muscles were exposed As was to be expected touch of the skin resulted in high amplitude afferent volleys in the trigeminal fibers and these were taken as proof of satisfactory recording conditions In most filaments no spontaneous activity from skin afferents was present and this eliminated the risk that background activity from exteroceptors might mask a possible afferent inflow from muscle receptors Stretch or touch of the muscle bellies or i v injections of succinylcholine (*cf* Granit *et al* 1953) did not give rise to any afferent activity whatever in the filaments of the facial or trigeminal nerves No evidence pointing to the presence of mechanoreceptors in the facial muscles could thus be found

Discussion

The reflexes set up in the facial muscles in anesthetized cats in response to taps on the face bear many resemblances to those obtained in similar experiments on man (Kugelberg 1952) In both species two successive responses can be evoked their thresholds are similar they both show a local sign and the latency of the secondary response varies over a wider range than that of the primary response But the investigations have also revealed differences between the two species Thus the duration of the late response is much briefer in cat than in man and in the cat the receptive field of this response is smaller than that of the early response whereas in man the late reflex component can be evoked over larger areas Since our experiments were performed on anesthetized cats and the secondary response is most likely relayed through a complex interneuron chain the differences thus observed need however not necessarily mirror actual dissimilarities in the functional organization of the reflex pathways in cat and man

In the present investigation special interest was paid to the early reflex component in order to ascertain whether it might be of proprioceptive origin In favor of such a concept are the findings that a tap on the skin close to the muscle was more effective than one applied at a more remote area and that the latency of the early reflex component varied over a very narrow range (*cf* Kugelberg 1952) However from a number of other observations it seemed less likely that the reflex should be proprioceptive Thus (*1*) the amplitude of the early response was the same whether the reflex was elicited by a tap on the intact skin overlying the muscle or from a skin flap (2) when the stimulus was applied directly to the muscle belly no early response was produced (*3*) measurements of the central relay time indicate that the early response is mediated through a polysynaptic reflex arc and finally (*4*) recordings from peripheral filaments of the trigeminal and facial nerves revealed that no afferent fibers deriving from muscle spindles were present

As mentioned in the introduction studies of the blink reflex in man have suggested that the primary reflex discharge should be a myotatic reflex (Kugelberg 1952). In a recent preliminary report (Shahani and Young 1968) it has been shown that in man both the early and the late components are set up in the orbicularis oculi on intradermal stimulation and it was suggested that both components are 'cutaneous reflexes'. Since the possibility exists that the trigeminal nerve in man contains proprioceptive filaments that are activated by an intradermal stimulus the findings by Shahani and Young are, however, not incompatible with the concept that the early reflex component in man is a monosynaptic reflex originating in muscle spindles. Nor are the results obtained in the present investigation necessarily inconsistent with Kugelberg's original interpretation since species differences with respect to the presence of muscle spindles have often been observed, *e.g.* in tongue muscles (Cooper 1953, Blom 1960).

The secondary reflex component could be shown to be of exteroceptive origin and to be transmitted through a separate long latency reflex arc, this is in agreement with previous results from experiments on man (Kugelberg 1952) and on cat (Tokunaga *et al.* 1958). Two other mechanisms may, however, also be engaged in the elicitation of the secondary response. Thus an off volley may be set up by reactivation of skin receptors when the stimulus probe is withdrawn from the skin, and if the stimulus is brief enough this off volley may, via a short latency reflex transmission, coincide with *the secondary reflex component initiated at the onset of the stimulus.* This concept gains support from the fact that the reflexes evoked by brief stimuli were of higher amplitude than those set up on more long lasting stimulation. Furthermore, as shown in Fig. 7, a directly evoked contraction gives rise to a reflex response in the muscle set up by activation of skin receptors. The interval between the direct muscle action potential and the reflex potential is about 15 msec which corresponds to the interval between the two reflex components produced by a tap stimulus. Since skin receptors may also be activated in response to a reflex contraction (Lindquist and Mårtensson unpublished results) it follows that on a tap stimulus the contraction set up by the early reflex discharge may contribute to the late reflex discharge through an afferent volley from skin receptors.

The experiments described above show that the facial nerve contains high threshold afferent fibres which when stimulated may elicit a long latency reflex discharge in the orbicularis oculi and also facilitate transmission of a short latency reflex response in the same muscle set up by stimulation of trigeminal afferents. However recordings from peripheral branches of the facial nerve show that no afferent fibers from mechanoreceptors in the facial muscles occur in this nerve and hence afferent activity in the facial nerve cannot contribute to the reflexes set up in the cat on a tap stimulus applied to the face. The calibers of these afferent fibers might very well correspond to the range of 1—6 μ found by Bruesch (1944) for afferent fibers in the facial nerve. On electrical stimulation of afferent fibers in the cat's facial nerve Widen (1955) recorded evoked responses in the cerebellum the threshold [illegible] which was six times that of the cat's motor fibers, these responses were abol[illegible]

section of the vagal root and Widén concluded that these afferent fibers were nociceptive because the "evoked responses" were accompanied by dilatation of the pupil and changes in blood pressure High-threshold afferent fibers with reflex connections to muscles supplied by cranial nerves have previously been demonstrated in the hypoglossal nerve with electrophysiological techniques (Lindquist and Mårtensson 1969 b), and the afferent fibers of the two nerves may possibly serve similar functions However, before the physiological function of these afferent fibers and the significance of their reflex connections can be clarified, further research is necessary

The present experiments do not provide any evidence indicating that the cat's facial muscles should contain a proprioceptive reflex mechanism originating in muscle spindles The question arises whether the muscles might be served by a feedback from other afferent systems Studies of the dog's intrinsic laryngeal muscles have demonstrated that they do not contain any muscle spindles and that the gradation of the contraction may be governed by a feedback deriving from mechanoreceptors located outside the muscles mainly in ligament structures and around joints (Mårtensson 1963 1964 1968 *cf* also Kirchner and Wyke 1964 and Kirchner and Suzuki 1968) Results have been presented which suggest that the cat's intrinsic tongue muscles are served by a proprioceptive feedback from receptors in the lingual mucosa (Blom 1960, Porter 1966 Lindquist and Mårtensson 1969 b) The fact that a contraction of the orbicularis oculi can give rise to a high amplitude reflex response in the muscle through activation of skin receptors (*cf* Fig 7) suggests that exteroceptive receptors may serve a proprioceptive function in the reflex control of the facial muscles Also in man a similar arrangement may be operative regardless of whether muscle spindles are present since stimulation of an intact branch of the human facial nerve results in a reflex response in the orbicularis oculi of a latency which when taking into account the different peripheral conduction times in the two species is comparable to that in the cat (Lindquist unpublished observations)

References

Baum J Beitrage zur Kenntnis der Muskelspindeln *Anat Hefte* 1900 *13* 251—305
Blom S Afferent influences on tongue muscle activity *Acta physiol scand* 1960 *49* Suppl 170
Bowden R E M M F Lucas Keene M Gooding T Y Mahran and J I Withington The afferent innervation of facial and laryngeal muscles *Anat Rec* 1960 *136* 168
Bruesch S R The distribution of myelinated afferent fibers in the branches of the cat's facial nerve *J comp Neurol* 1944 *81* 169—191
Cooper S Muscle spindles in the intrinsic muscles of the human tongue *J Physiol* Lond) 1953 *122* 193 202
Ekbom K A B Jernelius and E Kugelberg Perioral reflexes *Neurology* (Minneap) 1952 *2* 103 111
Gandiglio G and L Fra Further observations on facial reflexes *J Neurol Sci* 1967 *5* 273—285
Granit R S Skoglund and S Thesleff Activation of muscle spindles by succinylcholine and decamethonium The effect of curare *Acta physiol scand* 1953 *28* 134 151
Haapanen L E A high sensitivity capacitance meter *Electronic Engng* 1962 *34* 183—185
Kadanoff, D Die sensiblen Nervenendigungen in der mimischen Muskulatur des Menschen *Z Mikrosk anat Forsch* 1956 *62* 1 15

KIRCHNER J A and M SUZUKI Laryngeal reflexes and voice production *Ann N Y Acad Sci* 1968 *155* 98—109

KIRCHNER J A and B WYKE Electromyographic analysis of laryngeal articular reflexes *Nature* (Lond) 1964 *203* 1243—1245

MÅRTENSSON A Reflex responses and recurrent discharges evoked by stimulation of laryngeal nerves *Acta physiol scand* 1963 *57* 248—269

MÅRTENSSON A Proprioceptive impulse patterns during contraction of intrinsic laryngeal muscles *Acta physiol scand* 1964 *62* 176—194

MÅRTENSSON A The functional organization of the intrinsic laryngeal muscles *Ann N Y Acad Sci* 1968 *155* 91—97

NILSSON B Y Structure and function of the tactile hair receptors on the cat's foreleg *Acta physiol scand* 1969 *77* 396—416

PORTER R Lingual mechanoreceptors activated by muscle twitch *J Physiol* (Lond) 1966 *183* 101—111

RUSHWORTH G Observations on blink reflexes *J Neurol Neurosurg Psychiat* 1962 *25* 93—108

SHAHANI B and R R YOUNG A note on blink reflexes *Proc Physiol Soc* 1968 C 15

TOKUNAGA A M OKA T MURAO H YOKIO T OKUMURA T HIRATA Y MIYASHITA and S YOSHITATSU, An experimental study on facial reflex by evoked electromyography *Med J Osaka Univ* 1958 *9* 397—411

WIDEN L Cerebellar representation of high threshold afferents in the splanchnic nerve *Acta physiol scand* 1955 *33* Suppl 117

Acta physiol. scand. 1970. 80. 160—171

From the Institute of Medical Physiology B, University of Copenhagen, Denmark

Renal Reabsorption and Metabolic Conversion of Galactose in the Cat

By

S. Nybo Rasmussen

Received 15 February 1970

Abstract

Rasmussen, S. Nybo. *Renal reabsorption and metabolic conversion of galactose in the cat.* Acta physiol. scand. 1970. *80.* 160—171.

Extrahepatic elimination of galactose occurs mainly in the kidney. Urinary excretion as well as metabolic conversion may be of significance. At plasma concentrations of galactose between 60 and 1200 mg per liter in cats urine to plasma concentration ratios below one were never observed. At low filtered loads the reabsorption fraction was close to 1.0. At higher loads the reabsorption fraction decreased and seemed to converge towards 0.6. The rate of metabolic conversion of galactose in the kidney — calculated as the RBF times the arterio-venous concentration difference minus the excretion rate — exceeded considerably the reabsorption rate at low filtered loads. At increasing loads the metabolic rates approached maximum values between 50 and 100 μg per minute and gram kidney weight. The capacity of the kidney for metabolic conversion of galactose appeared (per gram organ weight) to be in the same order of magnitude as that of the liver. Push flow experiments indicated that reabsorption of galactose takes place exclusively in the proximal tubular system. When at low plasma concentrations the metabolic rate exceeds the reabsorption rate the intracellular concentration of galactose in the tubular wall cells may stay low and this may promote diffusion into the cells and account for the high reabsorption fractions found (conversion reabsorption). At higher concentrations where the reabsorption rate exceeds the metabolic rate, reabsorption is probably due increasingly to carrier facilitated transcellular backdiffusion.

Galactose is predominantly eliminated from the organism by the liver, a fact underlying the clinical use of galactose tolerance tests as a measure of hepatic metabolic capacity (Waldstein *et al.* 1960, Tygstrup 1966, Tengström 1968 and Keiding 1968).

Extrahepatic elimination of galactose occurs mainly in the kidney (Bollman, Mann and Power 1935, Levine *et al.* 1950). Excretion of galactose in the urine as well as metabolic conversion within the kidney may be of significance since kidney tissue has been shown capable of metabolizing galactose (Krane and Crane 1959, Topper, Maxwell and Pesch 1960).

The mechanism of urinary excretion of galactose is not clearly understood. Increasing clearance of galactose with increasing plasma concentrations has recently been demonstrated in man (Tengström 1968). Earlier results obtained in the cat

(Gammeltoft and Kjerulf-Jensen 1943) and in man (Tygstrup 1961) indicated a similar relationship Other investigations carried out in dogs (Dominguez and Pomerene 1944, Eiler, Althausen and Stockholm 1944) revealed a fairly constant renal clearance of galactose within a wide range of plasma concentrations

The *total renal capacity for metabolic conversion* of galactose has not been measured and its significance for the reabsorption process has not been taken into consideration As pointed out by Kruhøffer and Nissen (1963) in their work on the handling of glycerol in the feline kidney, a metabolic conversion of a substance taking place within the tubular cells may promote the tubular reabsorption of this substance by keeping its intracellular concentration at a low level, and thus giving rise to a diffusion of the substance from the tubular lumen into the cells (a so-called conversion reabsorption) The purpose of the present study was to measure the renal capacity for metabolic conversion of galactose, to throw light on the tubular handling of galactose and, especially, to evaluate the possible extent of a "conversion reabsorption" Finally because of the use of the maximal galactose elimination rate as a liver function test it is necessary to determine the total rate of renal elimination relative to the rate of hepatic elimination of galactose at different plasma concentrations

Methods

All experiments were carried out in male cats with a mean weight of 4 25 kg (range 2 86—5 41 kg) The animals were anesthetized by chloralose 80—100 mg per kg injected i p as a 1 % solution and usually heparinized by i v injection of 2500 I U of heparin

During the whole experiment the cat was lying supine on an operation table heated thermostatically to maintain a rectal temperature of 37° C

an urethral catheter inserted via a temporary opening in the apex of the bladder The expanded end of the catheter was fixed in the internal urethral orifice by means of a ligature around the urethra Emptying of the bladder was brought about either by manual compression or by insufflation of air from a syringe connected to the slightly elevated apex of the bladder

flow (RBF) times the renal arteriovenous deficit of galactose In periods characterized by very constant concentrations of galactose in blood the difference between the rate of extraction from the blood and the rate of excretion in the urine was taken as a measure of the rate of renal metabolism.

At the end of the experiments the kidneys were taken out and weighed after removal of the surrounding fat and connective tissue (fascia renalis)

Measurement of the RBF Great accuracy is required when the RBF enters into a calculation of the rate of extraction of a substance from the blood A direct method using a flowmeter is preferable to indirect methods based upon the Fick principle The differential

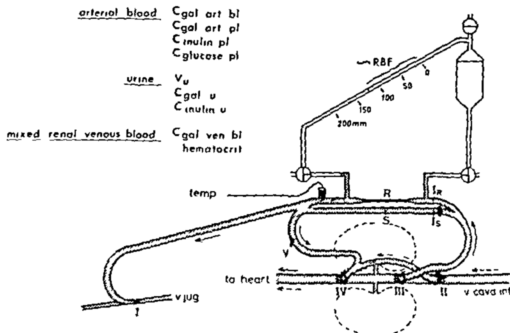


Fig 1 The experimental set up including the differential pressure flowmeter for measurement of the total renal venous blood flow (RBF) and (at the upper left) the quantities determined on samples of blood and urine R is the resistance tube, S a shunt → indicates mixed renal venous blood ⟶ indicates non renal venous blood Zero flow is recorded when the inlet I_R is clamped, flow is recorded when I_S is clamped Interruption of the RBF is avoided by performing the cannulations in the sequence indicated by the numbers I II, III and IV

pressure technique contributes one of the simplest and most precise methods The present version is described in detail elsewhere (Nybo Rasmussen 1970)

The inferior caval vein is isolated from about 5 cm distal and 3—4 cm proximal to the renal veins and all venous inlets apart from these are ligated The outflow from this caval segment ... include the flowmeter (Fig 1) ... vein and partly through the upper ... blood by passed from the distal ... displaced by a saline filled oblique manometer The deflection of the column is continuously recorded on a moving film At a normal RBF of 100 ml per minute and with blood of ordinary viscosity the pressure drop is about 40 mm of saline An increase in the renal venous pressure of this magnitude is not considered to interfere significantly with the functions of the kidney There were no significant differences in GFR between experiments with and experiments without measurement of the RBF In experiments where clearance measurements were performed both before and after connection of the flowmeter to the animal no significant reductions of the clearance of inulin were observed and the PAH extraction fractions were about 0 9 before as well as after these cannulations

The flowmeter must be calibrated with blood of the same viscosity as during blood flow measurements Calibration is performed with blood from the experimental animal adjusted to the temperature and hematocrit determined during the experiment The estimated overall error of the blood flow measurements is less than 2—3 per cent of the true value

Infusions After a priming dose constant infusions of galactose and inulin in 0 9 % saline were given and after a stabilization period of about 45 min essentially constant arterial concentration levels were obtained

The priming dose of galactose was calculated corresponding to a volume of distribution of 20—25 % of the body weight (Tygstrup 1966)

The rate of the sustaining administration of galactose was chosen according to the following principles [1] When plasma galactose concentrations below 300—400 mg/l were desired the rate o ... 'asma flow rate (average rate c ... w), Handbook of Biologica ... he hepatic extraction is close ... was desired to establish higher plasma concentration levels the rate of administration was chosen as the sum of an

In a few experiments the urine flow rate was increased by additional infusion of mannitol or Na_2SO_4

Heparinized blood from a donor cat was infused throughout the experiments to compensate for the blood samples drawn and for the small loss from bleeding

Analyses

G ...

b ...

g ...

stion 1 00) The recovery in whole blood was examined systematically at different concen- ... overy was found to fall ... 40 % it amounted on ... ie factor, 100/97 5 was ... i plasma and urine was ... n the formula

$$S = \sqrt{\frac{\sum d^2}{2n}}$$

where d is the difference between the two results of a duplicate determination and n the number of duplicate determinations The coefficient of variation was 0 9—1 0 % (2n=126) in plasma determinations, 1 3 % (2n=54) in determinations on whole blood

Glucose was analyzed enzymatically by means of a glucose oxidase method which is highly specific for glucose (Bergmeyer 1963, pp 123—130) The coefficient of variation was 1 5 % (2n=64)

Inulin was determined by the method of Bojesen (1952) using 1/5 the volumes of the original procedure The coefficient of variation was 0 8 % (2n=62)

Results

The following results were obtained from 61 clearance periods of 10—15 min duration performed in 13 different expts In 8 of these expts the rate of renal blood flow was measured and the results from 36 clearance periods allowed calculation of the rate of metabolic conversion of galactose In 3 of the remaining 5 expts, where RBF was not measured the circulation of the liver was excluded Neither the procedure of flow measurement by the differential pressure technique nor the evisceration and exclusion of the liver seemed to influence the glomerular filtration rate

The rate of glomerular filtration was on an average 8 7 ml/min (range 3 9—21 7) or 17 5 ml/(min · 100 g kidney) (range 8 5—24 2) (The results of two expts with very low GFRs are here excluded) The RBF was on an average 91 ml/min (range 42—136) or 176 ml/(min 100 g kidney) (range 130—284)

In Fig 2 is shown the time course of a single experiment with 5 clearance periods

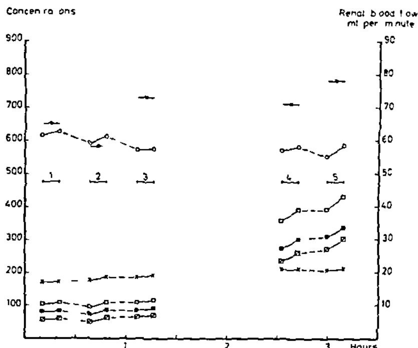


Fig 2 The time course of a single experiment with 5 clearance periods *Right ordinate* —● RBF (ml/min) *Left ordinate* □ $C_{gal\ art\ pl}$ (mg/l) ■ $C_{gal\ art\ bl}$ (mg/l) □ $C_{gal\ ven\ bl}$ (mg/l) ○ $C_{inulin\ pl}$ (mg/l × $C_{glucose\ pl}$ (mg/0 1 l)

The first 3 periods were performed at a galactose concentration level in arterial plasma of about 100 mg/l the last 2 periods at a level about 400 mg/l The results of this experiment are shown in Table I At both concentration levels the reabsorption fraction was about 0 90 Hence, nearly all galactose extracted from the blood was metabolized in the kidney and the metabolic rate exceeded greatly the reabsorption rate

The results from all experimental periods appear in Fig 3 which shows at various filtered loads the reabsorption rate and the metabolic rate all quantities expressed as μg galactose per minute and gram kidney weight Each point represents one clearance period

The urine was never free of galactose and urine to plasma concentration ratios below 1 0 were never observed The *rate of reabsorption* of galactose steadily increased with rising levels of the filtered load At low filtered loads — up to 50 μg/min·g kidney corresponding to arterial plasma concentrations of about 350 mg/l) — the reabsorption fraction (rate of reabsorption/rate of filtration) was almost constant and close to one (average 0 8) At higher loads the reabsorption fraction decreased apparently converging towards a value of about 0 6

TABLE I Calculation of results from a single experiment (cf. Fig. 2) Cat 4.35 kg

period	1	2	3	4	5
RBF (ml/min)	65	58	73	71	78
V_u (ml/min)	0.089	0.156	0.210	0.132	0.128
GFR (ml/min)	4.3	8.0	6.9	6.4	5.5
$C_{gal\ art\ pl.}$ (mg/l)	105	101	111	374	410
$C_{gal,\ art\ bl.}$ (mg/l)	81	78	89	286	321
$C_{gal,\ ven\ bl.}$ (mg/l)	58	55	67	243	285
$C_{gal\ art.bl.-ven\ bl.}$ (mg/l)	23	23	22	38	36
extraction fraction ($C_{gal.art.bl-ven\ bl.}/C_{gal.art.bl.}$)	0.29	0.30	0.25	0.13	0.11
filtration rate (mg/min) ($C_{gal\ art\ pl} \cdot GFR$)	0.44	0.81	0.77	2.38	2.23
excretion rate (mg/min) ($C_{gal\ u} \cdot V_u$)	0.05	0.07	0.08	0.26	0.26
reabsorption rate (mg/min) (filtration-excretion)	0.40	0.74	0.69	2.12	1.97
extraction rate (mg/min) ($C_{gal\ art.bl-ven\ bl} \cdot RBF$)	1.50	1.34	1.59	2.73	2.80
metabolic rate (mg/min) (extraction-excretion)	1.45	1.27	1.51	2.47	2.54
reabsorption fraction (*reabsorption/filtration*)	0.89	0.91	0.90	0.89	0.83

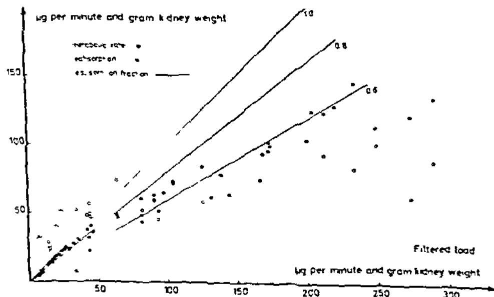


Fig. 3. The metabolic *rate* and the reabsorption rate at varying filtered loads of galactose. Results from 13 experiments, each point represents one *clearance period*. All quantities are related to the total weight of both kidneys remove

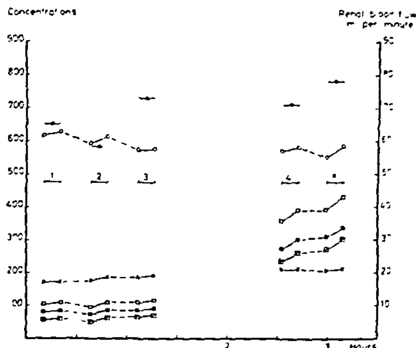


Fig 2 The time course of a single experiment with 5 clearance periods. *Right ordinate* ● RBF (ml/min). *Left ordinate* □ $C_{gal\ art\ pl}$ (mg/l) ■ $C_{gal\ art\ bl}$ (mg/l) □ $C_{gal\ ven\ bl}$ (mg/l) ○ $C_{inulin\ pl}$ (mg/l) × $C_{glucose\ pl}$ (mg/0.1 l)

The first 3 periods were performed at a galactose concentration level in arterial plasma of about 100 mg/l the last 2 periods at a level about 400 mg/l. The results of this experiment are shown in Table I. At both concentration levels the reabsorption fraction was about 0.90. Hence nearly all galactose extracted from the blood was metabolized in the kidney and the metabolic rate exceeded greatly the reabsorption rate.

The results from all experimental periods appear in Fig 3 which shows at various filtered loads the reabsorption rate and the metabolic rate all quantities expressed as μg galactose per minute and gram kidney weight. Each point represents one clearance period.

The urine was never free of galactose and urine to plasma concentration ratios below 1.0 were never observed. The *rate of reabsorption* of galactose steadily increased with rising levels of the filtered load. At low filtered loads — up to 50 μg/min·g kidney corresponding to arterial plasma concentrations of about 350 mg/l) — the reabsorption fraction (rate of reabsorption/rate of filtration) was almost constant and close to one (average 0.8). At higher loads the reabsorption fraction decreased apparently converging towards a value of about 0.6.

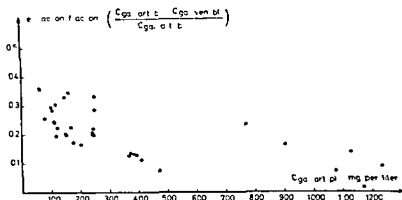


Fig 5 The extraction fraction of galactose (arterio-venous concentration difference for whole blood/concentration in arterial whole blood) versus arterial plasma concentration of galactose

tions of galactose in the range 61—394 mg/l) that on an average 22 % of the infused amount of galactose is extracted by the kidney

A comparison of the ability of the two organs to metabolize galactose is interesting A crude estimate of the rate of hepatic elimination per gram tissue in these periods is obtained by dividing the difference between the rate of infusion and the rate of renal extraction of galactose by the liver weight (tabular value) By doing so it appears that the metabolic rates calculated per gram total organ weight are in the same order of magnitude for the liver and the kidney(s) (the average metabolic rates in μg/(min g organ) being 60 and 41 resp) Relating the results to the weights of the tissue cells actually accounting for the metabolic conversion would of course be more reasonable but unfortunately not practicable

In Fig 4 is shown the *rate of reabsorption* of galactose per gram kidney weight versus the arterial plasma concentration The same results as shown in Fig 3 are here divided into three groups according to the magnitude of the corresponding GFR values (expressed per g kidney weight) It appears that in this range of arterial plasma concentrations (60—1200 mg/l) the reabsorption is still increasing with rising plasma concentrations It is obvious that at a certain plasma concentration the reabsorption rate is positively correlated to the magnitude of the GFR

In a few experiments the effect of osmotic diuresis on the reabsorption fraction for galactose was investigated The results indicated that osmotic diuresis is followed by a decrease in the reabsorption fraction *e g* in one experiment performed at arterial plasma concentrations between 800 and 900 mg/l a fall in the urine to plasma concentration ratio for inulin from about 45 (average from 6 control periods) to about 25 (3 periods) was produced by infusion of Na_2SO_4 This was accompanied by a decrease in the galactose reabsorption fraction from about 0.57 to 0.45

The plasma glucose concentrations were in most experiments between 1500

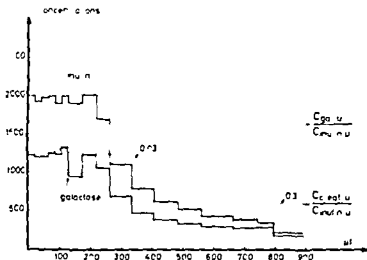


Fig 6 Push flow experiment *Abscissa* accumulated volume of urine sampled after the moment of intraaortic injection of 10 ml push solution (containing per liter 9 g NaCl 200 g mannitol 800 mg inulin 200 mg galactose and 20 g creatinine) Each plateau corresponds to a 5 sec sampling *Ordinate* urinary concentrations of galactose (mg per liter) and inulin ($mg \times 20^{-1}$ per liter) The concentrations of galactose and creatinine relative to those of inulin are given by the dotted lines (the zero level of the concentration fractions is the abscissa)

3000 mg/l (total range 700—3600 mg/l) The galactose reabsorption rate showed no correlation to the glucose concentration level within this range The urine was in most periods either free of glucose or contained only traces and the glucose content of the urine seemed unaffected by the variations in the galactose reabsorption rate observed in these experiments

Push flow experiments

The push flow technique (Aukland and Kjekshus 1966) was used in 5 separate experiments in order to elucidate where in the nephron the reabsorption of galactose takes place A strongly hypertonic solution of mannitol (usually 10 or 20 per cent) is rapidly injected into the abdominal aorta just above the renal arteries This causes an abrupt rise in the flow of urine before the injected mannitol appears in the urine The fluid standing in the tubules at the moment of injection is forced out at an increased rate and will not be modified to the usual extent by its further passage through the tubules one can get an idea of its initial composition — although as a somewhat blurred picture — by serial sampling and analyses of the urine from the ureter

Fig 6 shows the results of a push flow experiment performed at an arterial plasma concentration of galactose of 100 mg/l The *abscissa* is the accumulated volume of the urine in µl sampled after the moment of the injection The *ordinate* presents urinary concentrations and concentration ratios The rise in the *creatinine to inulin concentration ratio* corresponds to the first appearance of urine containing

mannitol from the injected push solution which also had a high concentration of creatinine The *first* samples represent the upper urinary tract and the distal parts of the nephron the *last* samples before the appearance of the new filtrate reflect the more proximal parts of the nephron The *galactose to inulin concentration ratio* is strikingly constant in most of the samples A significant increase in this ratio does not occur until such a volume of urine is accumulated that the creatinine to inulin concentration ratio has started to increase too A similar course of the galactose to inulin concentration ratio is consistent in the whole series of push flow experiments Results obtained by means of the push flow technique (as well as by the stop-flow technique) are less conclusive in regard to the functions of the proximal parts of the tubules in comparison with those of the distal parts of the tubules However, it seems safe to conclude that no reabsorption of galactose takes place in the more distal parts of the nephron and that the reabsorption process is located in the proximal part only The distal parts of the nephron must be considered impermeable to galactose

Discussion

The present studies do not allow any definite conclusion concerning the mechanism by which galactose is reabsorbed in the renal tubules but from the data obtained certain conclusions may be drawn (*cf* Fig 3)

The results give no direct evidence for active up-hill transportation because urinary concentrations of galactose lower than the corresponding plasma concentrations were never observed Complete exclusion of the occurrence of such transport can however only be provided by micropuncture studies Accumulation of galactose against a concentration gradient has been claimed by Krane and Crane (1959) and by Kleinzeller Kolinska and Benes (1967) in rabbit kidney cortex slices

Metabolic conversion of galactose has been shown to take place in cortical tissue by the same workers The precise site of the process is however not known Assuming that the metabolism takes place in the cells of the proximal tubular wall across which the reabsorption occurs the proximal tubular cells must be considered permeable to galactose at both the luminal and the basal side since at *low filtered loads* the metabolic rate exceeds the reabsorption rate Galactose must enter the cells from both the tubular lumen and the peritubular space (peritubular capillaries) The permeability for galactose seems greater at the luminal side than at the basal side because the rate of entry of galactose into the cells from the luminal side is of same order of magnitude as the rate of the simultaneously occurring entry from the basal side although the high fractional reabsorption of galactose (relative to water) implies that galactose is offered to the luminal membrane at lower concentrations than to the basal membrane

It seems unlikely that simple diffusion across the tubular wall can alone account for the high reabsorption fractions found because substances like mannitol which are of similar molecular weight and water lipid solubility as galactose are practically

not reabsorbed Rather a *facilitated* (carrier mediated) *diffusion* as demonstrated in the red cell membrane (Wilbrandt 1960, Lacko *et al* 1960) seems likely

A so called *conversion reabsorption* may play a role, especially at *low* filtered loads when the metabolic rate exceeds the rate of reabsorption The intracellular concentration of galactose in the tubular wall cells may thus stay low and this will promote diffusion into the cells and account for the relatively high reabsorption fractions found The fact that the reabsorption fraction decreases at plasma concentrations where the metabolic rate apparently approaches maximum (or, in other words becomes saturated supports the assumption that the metabolic conversion takes place in the same part of the tubule as the reabsorption

At *higher* loads where the reabsorption rate exceeds the metabolic rate reabsorption is probably increasingly due to carrier facilitated transcellular backdiffusion

The reabsorption fraction for galactose at higher plasma concentrations is probably of the same magnitude as the proximal reabsorption fraction for water and the fall in the galactose reabsorption fraction which may be produced by osmotic diuretics indicates that there is a close correlation between the galactose reabsorption and the water reabsorption

The present investigation revealed no upper limit for the galactose reabsorption rate in the range of arterial plasma concentrations up to 1200 mg/l A positive correlation between the reabsorption rate and the GFR corresponds to the findings of Deetjen and Boylan (1968) who in microperfusion studies on proximal tubules of the rat kidney showed an increment in *glucose* reabsorption with increments in the perfusion rate

)

References

AUKLAND K and J KJEKSHUS Tubular salt and water transport in hydrated dogs studied with push flow technique *Amer J Physiol* 1966 *210* 971—979

BERGMEYER H U *Method of enzymatic Analysis* Verlag Chemie Acad Press N Y and London 1963 pp 123—130

BOJESEN E A method for determination of inulin in plasma and urine *Acta med scand* 1952 *142* Suppl 266 275—282

*Bollman J L F C Mann and M H Power The utilization of galactose following com*plete removal of the liver *Amer J Physiol* 1935 *111* 483—491

DEETJEN P and J W BOYLAN Glucose reabsorption in the rat kidney *Pflugers Arch ges Physiol* 1968 *299* 19—29

DE VERDIER C H and M HJELM A galactose oxidase method for the determination of galactose in blood plasma *Clin chim Acta* 1962 7 742—744

DOMINGUEZ R and E POMERENE Kinetics of the disappearance of galactose from the plasma after a rapid intravenous injection *Amer J Physiol* 1944 *141* 368—381

EILER J J T L ALTHAUSEN and M STOCKHOLM Absorption of galactose by the renal tubules of the dog *Proc Soc exp Biol (N Y)* 1944 56 67—70

[illegible] The mechanism of renal excretion of fructose and *Acta physiol scand* 1943 6 368—384

[illegible]hiladelphia 1954

[illegible]he enzymatic determination of galactose in human [illegible] with galactose oxidase *Clin chim Acta* 1967 *15* 87—96

KLEINZELLER A J KOLÍNSKA and I BENEŠ Transport of glucose and galactose in kidney cortex cells *Biochem J* 1961 *104* 843—851
KRANE S N and R. K. CRANE The accumulation of D galactose against a concentration gradient by slices of rabbit kidney cortex *J biol Chem* 1959 *234* 211—216
KRUHOFFER P and O I NISSEN Handling of glycerol in the kidney *Acta physiol scand* 1963 *59* 284—294
LACKO L M BURGER L HEJMOVA AND J REJNKOVA *Exchange transfer of sugars in human erythrocytes* Symp membrane transp metab Proc (Prag) 1960 399—408
LEVINE R M S GOLDSTEIN B HUDDLESTUN and S P KLEIN Action of insulin on the "permeability of cells to free hexoses as studied by its effect on the distribution of galactose *Amer J Physiol* 1950 *163* 70—76
RASMUSSEN S NYBO Measurement of renal blood flow by a differential pressure technique (In press)
RELANDER A Intravenous galactose Tolerance Test. Normal Series of Children *Scand J clin Lab Invest* 1968 *22* 196—198
TENGSTROM B Enzymatic determination of glucose and galactose in urine *Scand J clin Lab Invest* 1966 *92* Suppl. 18 104—113
TENGSTROM B An intravenous galactose tolerance test and its use in hepatobiliary diseases *Acta med scand* 1968 *183* 31—40
TENGSTROM B Renal excretion of galactose in man with determination of the maximal tubular reabsorption for galactose *Scand J clin Lab Invest* 1968 *21* 321—326
TOPPER Y J E S MAXWELL and L. A PESCH On the mechanism by which progesterone stimulates galactose metabolism. *Biochim biophys Acta* (Amst) 1960 *37* 563—565
TYGSTRUP N The urinary excretion of galactose and its significance in clinical intravenous galactose tolerance tests *Acta physiol scand* 1961 *51* 263—274
TYGSTRUP N *Leverfunktionsbestemmelse med intravenøs galaktosebelastning* Danish thesis 1966
TYGSTRUP N and K. WINKLER Kinetics of galactose elimination *Acta physiol scand* 1954 *32* 354—362
WALDSTEIN S S L A GREENBURG A D BIGGS and L. CORN Demonstration of hepatic maximum removal capacity (L_m) for galactose in humans *J Lab clin Med* 1960 *55* 462—475
WILBRANDT W *The sugar transport across the red cell membrane* Symp membrane transp metab Proc (Prag) 1960 388—398

Acta physiol. scand. 1970. 80. 172—177

From the Department of Physiology, Karolinska Institutet Stockholm Sweden

Potentiation by Various Smooth Muscle Stimulants of an Isolated Sympathetic Nerve-Seminal Vesicle Preparation from the Guinea-Pig[1]

By

NILS O SJÖSTRAND and GORAN SWEDIN

Received 18 February 1970

Abstract

SJOSTRAND N O and G SWEDIN *Potentiation by various smooth muscle stimulants of an isolated sympathetic nerve-seminal vesicle preparation from the guinea-pig* Acta physiol scand 1970 *80* 172—177

The contractions of the seminal vesicle in response to hypogastric nerve stimulation or coaxial stimulation were recorded with a balloon inserted in the lumen of the seminal vesicle
Adrenaline (A) noradrenaline (NA) acetylcholine (Ach) histamine (Hi), serotonin (5-HT) barium chloride and high concentrations of angiotensin were found to potentiate the motor response of the seminal vesicle to sympathetic nerve stimulation This potentiation occurred with concentrations 10—100 times smaller than those giving a direct contraction of the organ Prostaglandins (PGE₁ PGF₂ and PGF₂α in low concentrations (1—10 ng/ml) exerted no effect or caused a slight decrease in the response to sympathetic nerve stimulation while higher concentrations 100 ng—1 µg/ml) enhanced the response to nerve stimulation. This effect was pronounced with PGE₁ but rather weak with the other prostaglandins Oxytocin, vasopressin and felypressin were without effect on the seminal vesicle preparation It is concluded that the seminal vesicle responds to smooth muscle stimulants in a similar way as the vas deferens

Various smooth muscle stimulants have been found to cause potentiation of the motor response of the guinea-pig vas deferens to hypogastric nerve stimulation Thus Sjöstrand (1961) Holman and Jowett (1964) and Sjostrand and Swedin (1968) reported potentiation by A NA and Ach Hi and 5-HT also exert this action (Sjöstrand 1961 Sjostrand and Swedin 1968) as well as angiotensin (Benelli *et al* 1964, Sjöstrand and Swedin 1968) bradykinin (Sjostrand and Swedin 1968), high concentrations of PGE₁ (Mantegazza and Naimzada 1965 Sjostrand and Swedin 1968, Euler and Hedqvist 1969) Substance P (Sjöstrand 1961 Sjostrand and Swedin 1968) and barium chloride (Sjostrand and Swedin 1968)

[1] A preliminary account of the present work has been given at the XIIth Scandinavian congress for Physiology in Gothenburg August 1969 Acta physiol scand Suppl 330, 72

In a recent study Naimzada (1969) reported that prostaglandins enhanced the motor response of the seminal vesicle to hypogastric nerve stimulation The present investigation was performed in order to see if also other smooth muscle stimulants, in low concentrations, increase the motor response of the seminal vesicle to sympathetic nerve stimulation

Material and methods

[illegible] eight of 700 g were used The guinea [illegible] icles were removed together with their [illegible] l bath containing Tyrode solution at [illegible] or activity of the seminal vesicle was [illegible] (length 20 mm width 3 mm) After squeezing out the content of the seminal vesicle, the balloon with its cannula was inserted in the lumen of the vesicle The cannula was fixed with ligatures around the neck of the seminal vesicle The pressure within the balloon was recorded with a Statham transducer and recorded on a Grass polygraph Before experiments were started the balloon pressure was adjusted to 5 cm H_2O

Preganglionic stimulation Stimulating platinum electrodes (2 mm apart) were placed on the hypogastric nerve 1 5—4 cm from the organ which means that the stimulation was mainly preganglionic (*cf* Sjostrand 1965 Naimzada 1966, Ferry 1967) The electrodes were kept in the bath The nerve was stimulated every one or two minutes for 5 sec with supramaximal [illegible]

[illegible] the seminal vesicle was insulated A second platinum electrode, insulated except for its last cm was then placed in the bath The supramaximal voltage was found to be 35—40 V The [illegible] with hypogastric nerve stimulation [illegible] and noradrenaline as bitartrate, acetyl [illegible] roxytryptamine (serotonin) as creatine [illegible] val[5] angiotensin (Hypertensin® Ciba), bradykinin (BRS [illegible] Sandoz) oxytocin (Syntocinon® Sandoz) lysinvasopressin (Sandoz), felypressin (Octopressin®, Sandoz)

Results

The seminal vesicles responded to the nerve stimuli by an increase in balloon pressure of about 10—30 mm Hg In many preparations a slight spontaneous activity generally with pressures less than 5 mm Hg was noticed

When the hypogastric nerve was stimulated at a distance of 1 5—4 cm from the organ hexamethonium (20 μg/ml) completely blocked the mechanical response of the seminal vesicle This was not the case when the nerve was stimulated close (less than 0.5 cm) to the organ or when coaxial stimulation was performed

Biogenic amines

Distinct direct contractions of the seminal vesicle was seen when high concentrations ($5\times10^{-5}-1\times10^{-4}$ M) of A NA, Ach and Hi were given To obtain even a moderate response to 5 HT very high concentrations ($1-5\times10^{-3}$) had to be used

A NA Ach and Hi in doses about 100 times smaller ($2\times10^{-7}-1\times$ [illegible] M) than those giving a clearcut direct response of the organ caused however, a conspicuous potentiation of the motor response to sympathetic nerve stimulation 5 HT in con



Fig 1

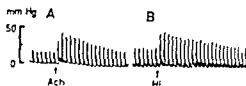


Fig 2

Fig 1 Isolated hypogastric nerve seminal vesicle preparation 80 V, 2 msec 10 imp/sec for 5 sec every second min a = noradrenaline 2×10^{-6} M b = noradrenaline 1×10^{-5} M c = direct effect of 2×10^{-4} M of noradrenaline W = washing

Fig 2 Isolated seminal vesicle preparation Coaxial stimulation A 35 V 2 msec 5 imp/sec for 5 sec every min Ach = 1×10^{-7} M acetylcholine B 35 V 2 msec 7 imp/sec for 5 sec every min Hi = 4×10^{-6} M histamine

centrations of $3-8\times10^{-4}$ M also caused a potentiation of the response to nerve stimulation but this was generally of a low magnitude

There were generally rather great differencies in sensitivity of different preparations to biogenic amines but in general they could be ranked in the following order Ach $>$ A $\lesseqgtr$ NA $<$ Hi $>$ 5 HT (Fig 1—3) The potentiation generally lasted about 10—15 min but disappeared almost immediately after washing

The potentiation was of the same magnitude with stimulating electrodes on different positions on the hypogastric nerve (0 5—4 cm from the organ) or with coaxial stimulation

Other smooth muscle stimulants

Barium chloride (0 08 -0 2 mg/ml Fig 4) exerted a marked potentiation on the seminal vesicle preparation which often after some time started spontaneous contractions between the periods of nerve stimulation



Fig 3



Fig 4

Fig 3 Isolated seminal vesicle preparation Coaxial stimulation A 35 V 2 msec 6 imp/sec for 5 sec every min Adr = 2×10^{-6} M adrenaline B 35 V 2 msec 8 imp/sec for 5 sec every min 5 HT = 5×10^{-4} M serotonin

Fig 4 Isolated hypogastric nerve seminal vesicle preparation 70 V 2 msec 5 imp/sec for 5 sec every min Ba Cl_2 = Barium chloride 0 1 mg/ml

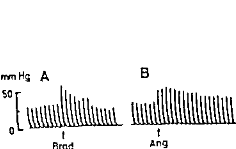


Fig 5

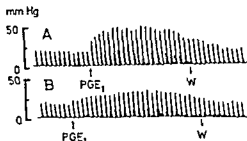


Fig 6

Fig 5 *A* Isolated hypogastric nerve seminal vesicle preparation 85 V, 2 msec, 6 imp/sec for 5 sec every min Brad = bradykinin 1 μg/ml *B* Isolated seminal vesicle preparation 7 imp/sec for 5 sec every min Ang = angiotensin 10 μg/ml

Fig 6 Isolated hypogastric nerve seminal vesicle preparation 80 V, 2 msec, 5 imp/sec for 5 sec every min *A* PGE1 1 μ/ml *B* PGE1 100 ng/ml W = washing

Distinct effects were also obtained by *bradykinin* (0 4—1 5 μg/ml) Fig 5 A, while *angiotensin* required high concentrations (10—200 μg/ml) in order to exert potentiation (Fig 5 B) This was the case in experiments with hypogastric stimulation as well as with coaxial stimulation

Prostaglandins (PGE_1 PGE_2 and $PGF_{2\alpha}$) in low concentrations (1—20 ng/ml) had no effect or exerted a slight inhibition of the response to sympathetic nerve stimulation, while higher concentrations (0 1—1 μg/ml) caused a potentiation In the case of PGE_1 it was pronounced and longlasting (Fig 6) while the other prostaglandins (PGE_2 and $PGF_{2\alpha}$) were less potent in causing a potentiation of the preparation *Oxytocin* *vasopressin* and *felypressin* in concentrations up to 0 2 units/ml were without effect

Discussion

In a previous report (Sjöstrand and Swedin 1968) potentiation of the guinea pig hypogastric nerve-vas deferens preparation to various smooth muscle stimulants on nerve stimulation was reported In the present study it has been shown that the smooth muscle stimulants (A NA Ach 5 HT histamine, angiotensin, bradykinin, different prostaglandins $BaCl_2$) exert a similar action on the isolated guinea pig seminal vesicle

The motor innervation of the seminal vesicle is probably adrenergic (Sjöstrand 1962 Falck Owman and Sjöstrand 1965, Naimzada 1966) The intraluminal pressure recording may give a more physiological picture of the contractions of the seminal vesicle than registration of the contractions of the longitudinal muscle layer only particularly since this muscle layer is rather poorly developed in the guinea-pig (Leydig 1850 Disselhorst 1904)

In comparison to the hypogastric nerve—vas deferens preparation the seminal vesicle seems to be less sensitive to 5 HT and angiotensin For the other biogenic amines the sensitivity appears to be about the same The finding that prostaglandins (PGF_{1}, PGE_{2} and PGE_{1}) in certain concentrations can potentiate the seminal vesicle preparation to nerve stimulation confirm the recent finding of Naimzada (1969) The inhibition occasionally seen with low doses of prostaglandins in the present study is in accordance with the effect on the vas deferens (Sjöstrand and Swedin 1968 Euler and Hedqvist 1969) and could be due to reduced NA release from the sympathetic nerve terminals (Hedqvist 1969 Hedqvist and Brundin 1969)

Regarding the possible mechanism for the potentiation (*cf* Sjöstrand and Swedin 1968) the present finding with coaxial stimulation apparently excludes an action on the peripheral ganglion cells (*cf* Sjöstrand 1965 Ferry 1967) and point at an action either on the adrenergic nerve terminal or on the smooth muscle cell In this respect angiotensin may diverge from the other substances used with part of its potentiating action on the ganglion cells (Sjöstrand and Swedin 1968) The insensitivity of the seminal vesicle to angiotensin makes this organ unsuitable for studies on the mechanism of action of this drug The site of action of the smooth muscle stimulants is however at least partly on the smooth muscle cell since it occurs also in electrically stimulated denervated vasa deferentia (Sjöstrand and Swedin to be published

This investigation has been supported by grants from Stiftelsen Magnus Bergvalls Minne and Lars Hiertas Minne The authors wish to thank Ciba A G and Sandoz A G for generously supply of drugs

References

BENELLI G D DELLA BELLA and A GANDINI Angiotensin and peripheral sympathetic nerve activity *Brit J Pharmacol* 1964 22 211—219

DISSELHORST R Ausfuhrapparat und Anhangsdrusen der mannlichen Geschlechtsorgane (*vierter Teil Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbeltiere Albert Oppel* 1904 Gustav Fisher Verlag Jena

EULER U S v and P HEDQVIST Inhibitory action of prostaglandins E_1 and E_2 on the neuromuscular transmission in the guinea pig vas deferens *Acta physiol scand* 1969 7 510—512

FALCK B CH OWMAN and N O SJÖSTRAND Peripherally located adrenergic neurons innervating vas deferens and the seminal vesicle of the guinea pig *Experientia* (Basel) 1965 21 98—100

FERRY C B The innervation of the vas deferens of the guinea pig *J Physiol* (Lond) 1967 *192* 463—478

HEDQVIST P Modulating effect of prostaglandin E_2 on noradrenaline release from the isolated cat spleen *Acta physiol scand* 1969 75 511—512

HEDQVIST P and J BRUNDIN Inhibition by prostaglandin E_1 of noradrenaline release and of effector response to nerve stimulation in the cat spleen *Life Sci* 1969 *8* 389—395

HOLMAN M and A JOWETT Some actions of catecholamines on the smooth muscle of the guinea pig vas deferens *Aust J exp Biol med Sci* 1964 *42* 40—53

LEYDIG F Zur Anatomie der mannlichen Geschlechts und Analdrusen der Saugethiere *Z wiss Zoologie* 1850 II

MANTEGAZZA P and M KABIR NAIMZADA Attività della prostaglandina E_1 sul preparato nervo ipogastrico-deferente di varie specie animali *Atti Accad med lombarda* 1965 *20* 58—64

NAIMZADA M K, *Response of the guinea pig isolated seminal vesicle to stimulation of the hypogastric nerve* *Med Pharmacol exp* 1966 *15* 561—567
NAIMZADA, M K Effects of some naturally occurring prostaglandins on the hypogastric nerve seminal vesicle preparation of the guinea pig *Life Sci* 1969 *8* 49—55
PATON W D M The response of the guinea pig ileum to electrical stimulation by coaxial electrodes *J Physiol* (Lond) 1955 *127* 40—41 P
SJOSTRAND N O, Effect of some smooth muscle stimulants on the motor response of the isolated guinea pig vas deferens to hypogastric nerve stimulation *Nature* (Lond) 1961 *192* 1190—1191
SJOSTRAND N O Effect of reserpine and hypogastric denervation on the noradrenaline content of the vas deferens of the guinea pig *Acta physiol scand* 1962 *56* 376—380
SJOSTRAND N O The adrenergic innervation of the vas deferens and the accessory male genital glands *Acta physiol scand* 1965 65 Suppl 257
SJOSTRAND N O and G SWEDIN Potentiation by smooth muscle stimulants of the hypogastric nerve vas deferens preparations from normal and castrated guinea pigs *Acta physiol scand* 1968 *74* 472—479
SJOSTRAND, N O, and G SWEDIN Potentiation by smooth muscle stimulants of an hypogastric nerve seminal vesicle preparation from the guinea pig *Acta physiol scand* 1969 *60* Suppl. 330

Acta physiol. scand. 1970 80. 178—195

From the Department of Physiology, Biological Institute University of Umeå Sweden

Single Unit Analysis of Mechanoreceptor Activity from the Human Glabrous Skin

By

M KNIBESTÖL AND A B VALLBO

Received 20 February 1970

Abstract

KNIBESTÖL, M and A B VALLBO *Single unit analysis of mechanoreceptor activity from the human glabrous skin* Acta physiol scand 1970 80 178—195

Single unit impulses were recorded from the median and the ulnar nerves in waking human subjects with percutaneously inserted tungsten electrodes 61 mechanoreceptor units in the glabrous skin were analysed with regard to the characteristics of their receptive fields and the basic physiological properties of their endings 4 different types of receptors could be distinguished This distinction was based mainly upon the adaptation and the receptive field characteristics although other differences were also observed The fields had very sharp borders for the majority of the slowly as well as the rapidly adapting units. The field sizes varied considerably between 10 mm² and 600 mm² For a minority of the receptors slowly as well as rapidly adapting ones the receptive fields were constituted of a center of high sensitivity and a wide surrounding area of lower sensitivity without distinct borders The four types of receptors encountered in the present study have striking similarities with four different types of mechanoreceptors which have been described in sub-human primates namely the type I and type II slowly adapting intradermal receptor according to Iggo's terminology an intradermal rapidly adapting receptor and the Pacinian corpuscle which is located in the subcutaneous tissues. Roughly 75 % of the units were slowly adapting This is a much larger proportion than found in the monkey Further it was shown how the recording method offers the opportunity to analyse the perceptive responses as well as the neurophysiological responses in first order neurons to mechanical stimuli

Single unit analysis of impulses from mammalian mechanoreceptors was introduced by Adrian and Zotterman (1926) in their studies of the receptors of the foot pads of the cat They found two principal types of responses one rapidly adapting and one more slowly adapting Later investigations have shown that these two principal classes of mechanoreceptors are not homogenous but each class is constituted of several types of receptors (e g Hunt and McIntyre 1960 Iggo 1966 Brown and Iggo 1967, Iggo 1968 Burgess Petit and Warren 1968 Iggo and Muir 1969) There are however, considerable differences among different species as to the types of receptors and their relative frequencies (Iggo 1966) Moreover there are even greater differences between different skin areas notably between hairy and non hairy skin Analyses of the mechanoreceptors in the glabrous skin of the monkey have been done

in several investigations (Iggo 1963, Lindblom 1965, Werner and Mountcastle 1965, Lindblom and Lund 1966 Mountcastle, Talbot and Kornhuber 1966 Talbot *et al* 1968) and it seems that there are at least 3 types of low threshold mechanoreceptors in this area one slowly adapting and 2 types of rapidly adapting receptors In man there are few corresponding investigations Hensel and Boman (1960) have studied single unit discharges from mechanoreceptors in hairy as well as non hairy skin of the hand Their recording method however, has the disadvantage that it requires surgery and cutting of the nerve, which make it unsuitable for studies on a larger scale

A relatively simple and atraumatic percutaneous method for recording of nerve impulses in man has been developed and it has been shown that it is suitable for studies of unitary discharges from skin mechanoreceptors (Vallbo and Hagbarth 1967, Vallbo and Hagbarth 1968) In the present investigation single unit analyses were made of mechanoreceptor activities from the glabrous skin in man with this recording method It will be shown that there are at least four different types of mechanoreceptors in this area The basic physiological properties of these receptors were similar to the properties of four types of mechanoreceptors which have been described in other mammals The relative number of slowly adapting receptors was much higher in the present material than in other materials from sub human primates

Methods

Thirteen experiments were carried out on 8 healthy male adults age between 20 and 35 years Recordings were made from the median and the ulnar nerves and in a few instances from the medial antebrachial cutaneous nerve The findings reported are based upon analyses [illegible]

block of warm modelling clay After cooling this block was used as a support for the subject s hand and fingers during the experiment In this position the palm and the volar aspects of the subject s fingers could be explored with mechanical stimuli and at the same time the nerves were readily accessible on the upper arm The recording electrode was inserted approximately 10 cm proximal to the elbow

The recording technique the recording and display systems were the same as those described in earlier reports (Vallbo and Hagbarth 1968 Vallbo 1970) The findings which constitute the basis for the discrimination between activity from cutaneous receptors and activity from muscle receptors have also been considered in some detail before (Vallbo and Hagbarth 1968 Hagbarth and Vallbo 1968 a 1968 b 1969)

Local mechanical stimulations of the skin were exerted with either of two probes made of Perspex The contact surfaces of the probes were flat circular and the areas were 1 0 and 10 mm² respectively The probes were connected to a small mechano-electrical transducer which the experimenter held with his hand Mechanical stimuli were delivered manually with this device to any desired skin area and an analogue signal of the force was recorded With [illegible] 1 mN (milli [illegible] and sustained [illegible] stretching of [illegible] g 8) In such cases the signal of the force was obviously just crudely related to the mechanical deformation at the point where the receptor was located Vibratory mechanical stimuli were delivered f[illegible] a commercial shaker (Pye Ling Model V 4) driven by a tone generator (Phillips type 2305 C) The peak force which could be obtained from this device when all movements

impeded was 2.83 newton. The maximum amplitude of the excursions varied with the frequency. To give a few examples, the peak-to-peak amplitude was 2.0 mm at 50 Hz and 0.4 mm at 200 Hz when the shaker was mechanically unloaded. The spring constant of the shaker was 0.51 mm per newton. A probe with a 1 mm² circular contact area was attached to the shaker. The experimenter handled the shaker manually and adjusted continuously the force against the skin surface to make the stimulus most effective for the particular receptor studied. With this device, the maximal frequency at which the receptor could be continuously entrained was measured, whereas [illegible] cal stimuli were [illegible] or tapping with a [illegible] surfaces and poking [illegible] probe. Care was ta[illegible] receptive fields were marked with a pencil on the skin surface. At the end of the experiment a transparent paper was placed over the receptive fields, the outlines of the fields were drawn onto this paper, and the sizes of the areas were later calculated.

Results

The present report is based upon recordings from 63 afferent units. The majority of the receptors (61) were located in the glabrous skin area of the hand. Fourty-one of the units were derived from the median nerve and 20 from the ulnar nerve. The two additional ones were located in the hairy skin on the volar aspect of the forearm. These two units were included for comparison with the glabrous skin units.

The conclusion that the endings were located in the skin and not in the deep structures was based upon same considerations as discussed in earlier reports (Vallbo and Hagbarth 1968, Hagbarth and Vallbo 1968 a, 1968 b, 1969). For many endings the nature of the single unit receptive fields and the low threshold for mechanical stimulation provided additional evidence for this conclusion (see p. 5 and p. 9).

Another 15 units were studied but they were not included in the present material for various reasons: it was uncertain whether the ending was a cutaneous mechanoreceptor or [illegible] the information concerning the properties of the ending was too fragmentary. Two of these units were possibly joint receptors as they could be activated by interphalangeal joint movements but not very easily by local pressure nor by voluntary contractions without joint movements. Another three spontaneously discharging units were possibly thermoreceptors as they exhibited a grouping of the impulses similar to that described by Iggo (1969) for thermoreceptors in primates and they were not affected by local mechanical stimuli.

The units were studied according to a program which included the following procedures: (a) determination of the receptor site and mapping of the receptive field by means of manually delivered mechanical stimuli, (b) qualitative determination of the adaptive characteristics of the receptor by mechanical stimuli, (c) measurement of the threshold for mechanical stimuli, (d) studies of the stimulus response relation during manually delivered local taps and sustained pressure, (e) determination of the maximum frequency at which the receptor could be entrained by vibratory stimuli and (f) studies of the relation between the nerve discharge and the subject's perceptive experience in response to mechanical stimuli. For all the units the procedures under (a) and (b) were performed whereas the whole program was carried out for only a few units. In these cases, the recording situation was stable enough to allow the recording of the single unit impulses for approximately 45 minutes.

TABLE I Frequency of occurrence of mechanoreceptors in the glabrous skin
Sample 61 units

Receptor type	No of units		% of sample		
Rapidly adapting units	15		24 6		
Units with distinct receptive field borders		14		23 0	Fig 2
Units with indistinct receptive field borders		1		1 6	Fig 4
Slowly adapting units	46		75 4		
Units with distinct receptive field borders		43		70 5	Fig 5
Units with indistinct receptive field borders		3		4 9	Figs 6, 7

The receptors could readily be classified into two principal groups rapidly adapting receptors which exhibited a discharge only when the stimulus was changing and slowly adapting receptors which exhibited a discharge also during a sustained pressure As will be shown below, several findings indicate that these two classes were not homogenous

Glabrous skin

The receptive fields of the endings in the glabrous skin areas were, for the majority of them located on the volar aspect of the hand and the fingers However, some of the fields extended to the lateral and the dorsal aspects of the fingers and a few of them were located all on the dorsal aspect of the end phalanges of the fingers Of the 61 receptors in the glabrous skin areas 46 units were slowly adapting and 15 units were rapidly adapting (Table I)

Rapidly adapting receptors All but one of the fifteen rapidly adapting units constituted a homogenous group as far as the present analysis is concerned whereas one receptor had clearly different properties The receptive fields of the units in the main group were very sharply demarcated a border could be determined with an accuracy of one or two dermal ridges Outside this border the threshold for mechanical stimuli increased abruptly From a transitional zone of a few mm very strong stimuli induced a weak discharge but in some cases it could be seen that these stimuli caused a deformation of the skin inside the borders as determined with much weaker stimuli Further away the stimulus was completely ineffective The distinct borders of the receptive fields were regarded as additional evidence for the conclusion that the afferent discharges originated from intradermal receptors The receptive fields are shown on a semi schematic drawing in Fig 1 A for 12 of the units in the main group It is seen that the fields were roughly rounded or oval in shape, and when located on the fingers the long axis was usually oriented in parallell with the

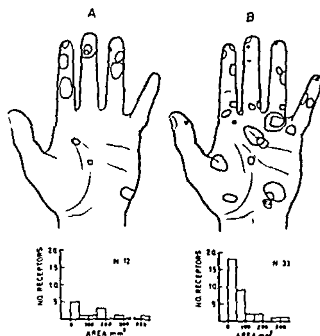


Fig 1 Semischematic drawings of 44 receptive fields (above) and histograms showing the distribution of receptive field sizes of the same units (below) A Rapidly adapting units B Slowly adapting units One slowly adapting unit included in the histogram is not shown in the drawing

long axis of the finger The sizes of the fields varied a lot from one unit to the other as is obvious from the drawing and from the histogram in Fig 1 A The smallest area was approximately 10 mm whereas the largest field which covered the whole volar aspect of the distal phalanx of the third finger was 620 mm in size The mean of the measured field sizes was 141 mm

An approximate estimate of the sensitivity of the endings to mechanical stimuli was obtained by measuring the minimum force required to evoke a single impulse when taps were delivered manually This method of stimulation implies that the rate of change of the stimulus was not controlled The threshold as measured in this way varied from one unit to the other between less than 1 mN and 600 mN Ten units were tested The threshold was less than 1 mN for one unit whereas it was between 1 and 10 mN for two units and above 10 mN for the remaining 7 units Fig 2 illustrates the responses of two units in this group to short mechanical stimuli of different peak forces and different rates of change of the force as shown by the analogue signal below the nerve records Most of the rapidly adapting receptors exhibited a discharge similar to that shown in Fig 2 B in that one or two occasionally three impulses appeared on the rising phase of the stimulus In some cases a few impulses also appeared on the falling phase A minority of the receptors exhibited a discharge more similar to that shown in Fig 2 A in that there was a longer train of impulses during the major part of the rising phase of the stimulus However, a clear distinction between these two types could not be demonstrated Similar types of responses from rapidly adapting mechanoreceptors have been described in



Fig 2 Responses of two rapidly adapting mechanoreceptors in the glabrous skin to taps of varying rates of rise Both units had distinct field borders The lower traces are analogue signals of the stimulating force given in newton (N)

the monkey (Talbot *et al* 1968) The records in Fig 2 also illustrate how the discharge was dependent upon the velocity of the indentation A minimum velocity was required in order to evoke a discharge and the interspike intervals were shorter the greater the velocity The critical slope was difficult to measure exactly with the stimulus system used but an estimate in three units indicated that it varied considerably (0 1—9 0 N/sec) Nine receptors were tested for their responses to vibratory stimuli The maximum frequency at which a unit could be continuously entrained in the sense that it discharged one impulse/cycle was determined when the frequency was changed in steps of 50 Hz The findings are presented in Fig 3 A where it can be seen that the frequency varied between 50 Hz and 500 Hz Although this was a very crude test the findings allowed a certain comparison between the rapidly and the slowly adapting receptors in the present study (see p 9)

One of the fifteen rapidly adapting receptors could clearly be distinguished by the nature of its receptive field from the units in the main group described in the previous sections (Fig 4) There was a small area in the middle of the palm where the sensitivity was high (black dot in Fig 4) The threshold to short taps was here less than 1 mN implying that this unit was more sensitive than 86 % of the total 36 mechanorepectors studied in this respect In Fig 4 A are shown representative discharges induced by mechanical stimulation of this central area It is seen that a series of impulses at high frequency was evoked during the skin indentation and one or two impulses when the stimulus was withdrawn The receptor could also be activated from a large area covering the whole palm and the four ulnar fingers (stippled area in Fig 4) The responses to short taps in the periphery of this area are shown in Fig 4 B It can be seen that only a few impulses were evoked by a

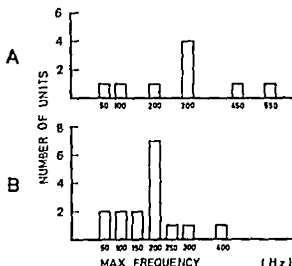


Fig 3 Diagrams showing the response to vibration of 25 mechanoreceptors Abscissa Maximum frequency at which the units could be entrained to respond with one impulse/cycle A Rapidly adapting units B Slowly adapting units

taps and that the threshold, as well as the critical slope were considerably higher This receptor was also tested for its response to vibration It could be entrained at a maximum frequency of 500 Hz when the stimulus was applied in the central area Thus this unit was one of the most responsive ones to high frequency stimulation as the maximum frequency was below 500 Hz for 92 % of the 26 units studied in this respect In the periphery of the receptive field the vibratory stimuli were not nearly as effective the unit could not be entrained at any frequency when the stimulus was applied more than 10 mm from the central area

Slowly adapting receptors Fourty-six receptors or 75 % of all the units in the glabrous skin of the hand exhibited a lasting discharge during a sustained pressure and they were accordingly classified as slowly adapting receptors Several findings indicated that these receptors did not constitute a homogenous group Fourty three of them had all similar properties whereas the remaining three units exhibited different characteristics (Table I)

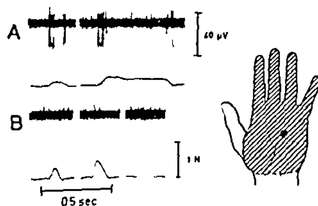


Fig 4 Responses of a rapidly adapting receptor with indistinct receptive field borders In the drawing the black dot indicates the point of maximal sensitivity and the stippled area the total receptive field A Responses to taps and sustained pressure at the point of maximal sensitivity B Responses to taps in the periphery of the field The lower traces are analogue signals of the stimulating force

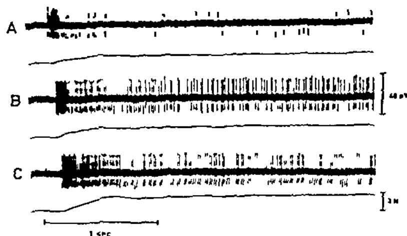


Fig 5 Responses of a slowly adapting receptor with distinct receptive field borders to stimuli of different intensities The ending was located on the volar aspect of the fifth finger on the middle phalanx The approximate stimulating force indicated by the lower traces and the mean impulse frequency in the steady state were in A 1 2 N and 28 imp/sec respectively in B 1 6 N and 37 imp/sec and in C 2 4 N and 48 imp/sec

A description will first be given of the units in the large group The receptive fields of these endings had similar properties as those described for the majority of the rapidly adapting receptors in that the fields were in general oval or rounded in shape and their borders were very distinct Remote stimuli or moderate stretching of the skin did not induce a discharge In Fig 1 B are shown on a semi schematic drawing the fields of 32 units The field areas were measured for these 32 receptors and for another one not indicated in the drawing They varied from 3 mm² to 340 mm² in size with a mean of 65 mm² A histogram of the fields sizes is shown in Fig 1 B The one receptive field not indicated in the drawing was located on the dorsal aspect of the distal phalanx of the fourth finger and it surrounded the nail This was the largest field in this group with an area of 340 mm² The difference in size between the receptive fields of the fast adapting units and those of the slowly adapting ones was not statistically significant ($P > 0.05$ Wilcoxon's t test) although the mean was higher for the fast adapting units

None of the units in the main group of the slowly adapting receptors was spontaneously active i e they were not discharging as long as local pressures were not exerted on the receptive field The responses of a representative unit to local sustained pressures of various intensities are shown in Fig 5 It is seen that the impulse frequency during the rising phase of the stimulus was high and dependent upon the rate of rise of the stimulus whereas the frequency during the sustained stimulus was much lower The impulse frequencies in response to strong static stimuli varied up to a maximum of 60 imp/sec from one receptor to another whereas during the rising phase of the stimulus frequencies of 500 imp/sec were seen for some of the

receptors It should be emphasized that these figures are not based upon a systematic exploration of the total frequency range of the units They merely give the order of magnitude As for the rapidly adapting units the thresholds to short taps delivered manually were determined for 25 of the slowly adapting receptors It varied from one receptor to the other between less than 1 mN and 100 mN For 12 % of the units (3) the thresholds were less than 1 mN whereas it was between 1 mN and 10 mN for 52 % (13) and above 10 mN for the remaining 36 % (9) The findings on this point suggest that there was not any pronounced difference between these slowly adapting units and the fast adapting units with regard to their sensitivities to this type of stimulus (see p 5) *The minimum force required to induce a sustained discharge was determined for 39 of these receptors It varied from one unit to another between 1 mN and 3 N* although it was less than 200 mN for the majority of them (85 %) The thresholds to taps were compared with the minimum force required to induce a sustained discharge for 24 units The sustained discharge thresholds were between two and ten times higher than the thresholds to taps for the majority (79 %) of them whereas no difference could be demonstrated for the remaining units with the method of stimulation used in the present study Sixteen of the slowly adapting receptors in this group were tested for their response to vibratory stimuli The maximal frequencies at which the receptors could be entrained were determined As can be seen in Fig 3 B this frequency varied between 50 Hz and 400 Hz It is also seen in Fig 3 that there was not any striking difference between the slowly adapting receptors and the rapidly adapting receptors in this material Thus there were several indications that the dynamic sensitivity was not very much different for these slowly adapting receptors and for the units in the main group of the fast adapting endings which both types were probably intracutaneous receptors

The remaining three slowly adapting receptors located in the hand had all properties which clearly distinguished them from the units in the main group described above Their receptive fields were not sharply delimited There was a central point or a small area not more than 3—10 mm² in size at which the sensitivity was maximal for local pressure and a wide surrounding field from which the receptor could be activated with stimuli of higher intensity The central area was located along the border of a nail for all the three receptors Another distinguishing feature was that they exhibited a discharge *in the absence of any stimulus* This spontaneous activity was very regular as can be seen in Fig 6 The impulse frequency varied from one receptor to the other between 1 and 20 imp/sec In addition the response to local mechanical stimuli was significantly different from the response of most of the units in the main group in that the impulse frequency was not very high during the rising phase of the stimulus (Fig 7) indicating that these receptors had a much lower dynamic sensitivity When a stimulus was withdrawn the unit was silent for a short period after which the spontaneous discharge gradually reappeared (Fig 6 and 7) The duration of the silent period was dependent upon the stimulus duration and intensity Thus there were three features which clearly distinguished these three receptors from the other slowly adapting ones the receptive fields were not sharply

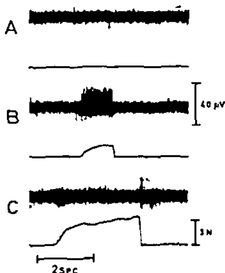


Fig 6 Discharges of a slowly adapting receptor with indistinct receptive field borders The ending was located at the very tip of the third finger A Spontaneous activity frequency 17 imp/sec. B Response to local pressure within the receptive field Note the pause after the stimulus C Response to local pressure on the finger pulp Note the high frequency initially after the stimulus The lower traces indicate stimulus time course and intensity

delimited the units were spontaneously discharging and their dynamic sensitivities were poor Possibly there was a fourth difference in that the discharge of the units in the main group was more irregular than the discharge of the three other units. However, additional analysis is required to establish this point The thresholds were not measured in detail for these three units but qualitative observations suggested that there was not any striking difference between the two types of slowly adapting receptors in this respect

The activity of these three units could be increased or decreased by a variety of mechanical stimuli which did not imply local pressure on the skin area overlying the apparent size of the receptor For instance stretching of the skin in the palm and movements of the distal interphalangeal joints were sufficient to modify the discharge of two of them Finger extensions increased the discharge whereas flexions decreased or completely inhibited the discharge For the third receptor a complex response could be demonstrated as illustrated in Fig 6 This receptor which had its maximal sensitivity area at the distal border of a nail right at the finger tip exhibited a regular spontaneous discharge with a frequency of 17 imp/sec (Fig 6 A) A local pressure on this area or close to it induced a marked increase of the discharge (Fig 6 B), whereas a local pressure on the volar aspect of the distal phalanx had the opposite effect (Fig 6 C) The spontaneous discharge was completely inhibited by this pressure and at the end of the stimulus the activity reappeared at a higher impulse frequency which gradually decreased to its original level This effect was seen from the larger part of the volar aspect of the distal phalanx but not from any other skin area In Fig 7 is shown the response of the receptor to a combined stimulation of the two skin areas The top trace shows the instantaneous impulse frequency During the time indicated by the bar below the records a pressure was applied on the volar aspect of the distal phalanx with a probe having an area of

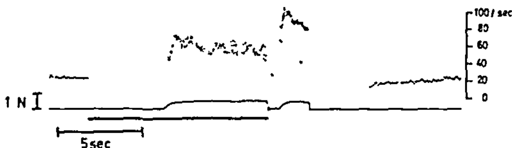


Fig. 7. Response of the same unit as in Fig. 6 to two different kinds of stimuli which one increased the discharge and the other one decreased the discharge. The record shows the *instantaneous impulse frequency of the unit and an analogue signal of the force by which a local* pressure was applied approximately at the point of maximal sensitivity at the tip of the third finger. The line below the record indicates the time during which a local pressure of approximately 2 N was applied with a probe of 2 cm² area on the finger pulp.

approximately 2 cm². It is seen that this resulted in a complete inhibition of the activity. While this stimulus was kept, a pressure was applied on the area of maximal sensitivity as indicated by the second trace from the top which is the analogue signal of this stimulus force. The joint effect was a discharge frequency of approximately 60 imp/sec. At the end of the bar the stimulus on the volar aspect of the finger was withdrawn and a stimulus of approximately the same intensity as before was applied after a short pause on the area of maximal sensitivity. This resulted in an impulse frequency of 80—100 imp/sec. Thus this receptor was capable of yielding an output which was a function of two simultaneous stimuli having opposite effects on its discharge. This test required that the subject voluntarily resisted the stimulating force in order to avoid joint movements. It could be shown that voluntary contractions by themselves did not affect the discharge of this unit nor was it particularly sensitive to joint movements. It could therefore be excluded that the observed effects were due to other factors than local mechanical stimulations of the tissue. The responses of these three units to remote mechanical stimuli could most easily be explained by assuming that the receptors were very sensitive to the amount of stretch of the skin. This property was one additional feature which distinguished them from the other slowly adapting units.

It could be shown for two of the three receptors in this group that they were sensitive to temperature. This was tested by pouring water of different temperatures on the skin. Warming (41° C) caused a complete abolition of the spontaneous discharge whereas cooling (11° C) increased the discharge frequency for a short period of one to two seconds.

The two types of slowly adapting mechanoreceptors in the glabrous skin of the human hand described in the present study have some similarities to the two types of slowly adapting receptors in the hairy skin of other mammals designated type I and type II by Iggo (Iggo 1966, Chambers and Iggo 1967, Iggo and Muir 1969). The type II slowly adapting receptor is spontaneously discharging and it has a high sensitivity to stretching of the skin whereas type I is not discharging unless mechani-

cal stimuli are applied on exactly defined points Further type II has a less pronounced dynamic sensitivity and a more regular discharge than type I These criteria are essentially the same as those which constitute the basis for a distinction between the two types of slowly adapting receptors in the present study

Hairy skin

Two mechanoreceptors in the hairy skin of the forearm were studied They will be briefly described as they seem to be of some interest in this context One of them had an oval receptive field with an area of approximately 50 mm² However this field was not homogenous but it was made up of several spots with considerably lower threshold to pressure and touch than the interjacent areas The exact number of spots could not be determined with the type of stimulation utilized which implied manual poking without control under microscope However there seemed to be at least 3 or 4 spots The threshold at these spots was very low approximately 1 mN the dynamic sensitivity of the ending was pronounced and the receptor was insensitive to stretching of the surrounding skin areas and it was not spontaneously discharging Thus, this unit resembled the type I slowly adapting receptor in the hairy skin of other mammals as described by Iggo with regard to the structure of the receptive field and the physiological characteristics of the receptor

The other unit had a receptive field which consisted of a spot of maximal sensitivity not more than 1 mm in diameter and an extensive skin area surrounding this spot The point of maximal sensitivity was located on the ulnar side of the forearm 10 cm proximal to the wrist (Fig 8) and the surrounding area from which the receptor could be activated included the greater part of the volar aspect of the forearm and the ulnar half of the hand The most effective stimuli in this area were stretching of the skin for which the receptor was extremely sensitive Even a minimal passive dorsal flexion of the wrist induced a few impulses probably as a result of stretching of the skin of the forearm The threshold at the central spot was 1 mN or less One striking property of this unit was that its activity could be affected in opposite ways by stretching of the skin in different directions This is illustrated in Fig 8 The arrows in the drawing indicate the directions in which the stretchings were applied and the dot indicates the point of maximal sensitivity In order to produce this kind of stimulus a pressure was exerted with the stimulating probe at an angle of approximately 45° to the skin surface The trace below the nerve records is an analogue signal of this pressure which is not supposed to represent a very accurate signal of the amount of stretch at the receptor site It is seen in the figure that a stretching in longitudinal directions elicited a conspicuous discharge during the stimulus This was followed by a silent period A stretching in the transverse direction inhibited the spontaneous discharge during the stimulus whereas an increased discharge appeared when the stimulus was withdrawn The opposite effects on the unit of stretchings in the different directions were probably accounted for by opposite mechanical effects which could be demonstrated in the test on the tissues at the receptor site As expected the skin was longitudinall[illegible]

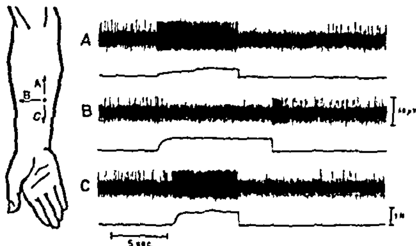


Fig. 8. Responses to skin stretch of a slowly adapting unit with indistinct receptive field borders in the hairy skin. In the drawing the black dot indicates the point of maximal sensitivity of the unit. The arrows indicate the directions in which the skin was stretched when the records in A, B and C respectively were obtained.

proximal and distal directions, whereas stretching in the transverse direction resulted in a marked longitudinal shortening of the skin. It seems therefore reasonable to assume that this ending was sensitive to elongation and shortening mainly in the longitudinal direction. Thus, this unit had several properties in common with the type II slowly adapting units described in the hairy skin of other mammals and also with the spontaneously discharging units in the human glabrous skin: the nature of the receptive field, the spontaneous discharge and the very pronounced sensitivity to stretching of the skin. Further, it was similar to the spontaneously discharging units in the glabrous skin in that the output of the receptor could be modified in two directions from a resting discharge by mechanical stimuli of different kinds.

Psychophysical relations

The recording technique used in the present study offers the opportunity to relate the neural activity in first order neurons to the sensations experienced by the subject in response to skin stimulations in one and the same experiment. In order to indicate the possible type of analysis the findings obtained in one test is presented in Fig. 9. Unitary nerve impulses were recorded from a slowly adapting receptor located on the volar aspect of the fifth finger. The unit had a threshold for taps of approximately 1.0 mN, implying that it belonged to the 14 % of the most low threshold mechanoreceptors in the glabrous skin as tested in the present study. Light stimuli were delivered manually with a piece of cotton wool to the centre of the receptive field and the subject was asked to report whether he could feel the stimulus or not and further, to give a rough estimate of the intensity of the stimulus. The number of spikes from this single unit elicited by each stimulus was related to the subject's

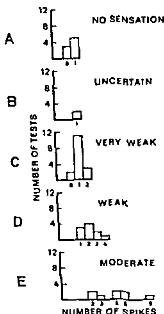


Fig 9 Responses of a low threshold, slowly adapting mechanoreceptor unit in the glabrous skin related to the subject's sensations Same unit as in Fig 5 Brief touches in the center of the receptive field of the unit with a piece of cotton wool For each stimulus the subject reported whether [illegible] estimation of the stimulus

report of sensation One histogram is shown for each of the five categories of subjective intensity estimation As can be seen there was a rough correlation between the number of impulses and the subject's intensity estimation of the stimulus, although there was a considerable scatter From the trials with stimulus intensities close to the subjective threshold (A—C) it can be seen that the subject reported no sensation in several cases (5) when a single impulse was elicited but in the majority of these cases (13) he was uncertain or he reported a weak sensation Whenever two impulses or more were elicited the subject experienced the stimulus without uncertainty As the unit had a very low threshold it seems justified to assume that the stimuli close to the threshold intensity for this unit did not induce conspicuous discharges from other mechano-repectors, although it can not be excluded that other endings were also excited Nevertheless the findings suggest that very few impulses, possibly one or two, could be sufficient to induce a sensation Hensel and Boman (1960) reach the same conclusion on the basis of neurophysiological recordings and psychophysical tests on humans in different experiments when the response to bending of a single hair in the hairy skin was analysed An analysis by Buchthal and Rosenfalk (1966) of the response to electrical stimulations at threshold intensities is largely in agreement with these findings

Discussion

Neurophysiological findings from animal experiments and psychophysical data obtained from man form the main basis of the current concept of the mechanisms in

volved in cutaneous sensibility When conclusions are based upon both types of information it is obvious that the validity of these conclusions is partly dependent upon to what extent the populations of receptors have the same properties in man and in the other animal studied Therefore physiological analyses of cutaneous receptors in man have implications beyond those of compartive neurophysiological studies in other mammals

In the present investigation unitary nerve impulses were studied with the purpose to characterize the basic physiological properties of mechanoreceptors in the human glabrous skin It is likely that the sample was strongly biased in favour of units with large diameter nerve fibres as the recording method probably does not allow the discrimination of unitary discharge in small fibres (Vallbo and Hagbarth 1968 Hagbarth *et al* 1970) The physiological properties of the mechanoreceptive end organs were probably of minor significance for the selection of the material as the methods of mechanical stimulation allowed great variations with regard to the intensity, the spatial extent and the time course of the stimulus Thus it is likely that the present sample was roughly representative of the mechanoreceptors with large diameter nerve fibres in the human glabrous skin of the hand except that there was probably a bias in favour of the most large diameter nerve fibre units

The receptors could clearly be separated in four different groups two types of rapidly adapting units and two types of slowly adapting ones The basis for the differentiation of the fast adapting units as analyzed in the present study was the nature of their receptive fields For one type of the fast adapting units the fields were small very sharply delimited and uniform with regard to the sensitivity These characteristics suggest that the endings were located in the skin and not in the subcutaneous tissues Units with the same physiological properties have been described in the glabrous skin areas of the monkey and it has been shown that the endings are located intradermally (Lindblom 1965 Talbot Darian Smith Kornhuber Mountcastle 1968) For the other type of fast adapting receptor encountered in the present study the receptive field consisted of a small central area of high sensitivity and a wide surrounding field without distinct borders from which the unit could be activated with higher stimulus intensities The same type of receptive fields have been described in the glabrous skin areas of the monkey (Lindblom and Lund 1966 Talbot *et al* 1968) In this animal it has been shown that the end organs are located in the subcutaneous tissues and further that the endings are very likely Pacinian corpuscles (Lindblom and Lund 1966)

The slowly adapting receptors could also be clearly separated in two groups One type of units had sharply delimited receptive fields and they were not spontaneously discharging Most of them had high dynamic sensitivities The same characteristics have been described for the majority of the slowly adapting receptors in the glabrous skin of the monkey (Iggo 1963 Lindblom 1965 Talbot *et al* 1968) The other type of slowly adapting receptor encountered in the present analysis had a lower dynamic sensitivity it exhibited a spontaneous discharge and it could be affected by mechanical stimuli from a large field without distinct borders although there was a small

central area with a maximal sensitivity. They were further particularly sensitive to stretching of the skin. These properties are very similar to those of the type II slowly adapting mechanoreceptor in the hairy skin of the cat, rabbit, monkey, and baboon (Iggo 1966, Chambers and Iggo 1967, Burgess, Petit and Warren 1968, Iggo and Muir 1969). However, the type II mechanoreceptor has not been clearly described in the primate glabrous skin although Iggo claims that it is present in this area (Iggo 1963, 1966). Further, a small percentage of spontaneously discharging mechanoreceptors has been encountered in the glabrous skin of the monkey (Mountcastle, Talbot and Kornhuber 1966). Considering these earlier findings it seems likely that the spontaneously discharging units encountered in the present investigation are identical with a type of receptor in the glabrous skin of the sub-human primates. The findings in the present investigation seem to supply additional evidence that the type II slowly adapting mechanoreceptor is present in the glabrous skin of primates. However, they seem to constitute only a minor fraction of the total slowly adapting receptor population in this skin area.

To summarize, four different types of mechanoreceptors were found in the human glabrous skin area in the present study and four types seem to be present in the same skin area of sub-human primates. In both types of investigations only large diameter nerve fibres were analysed. All available information indicate that each of the four types have in broad the same properties in man and monkey with regard to the organisation, size and shape of their receptive fields and their response to dynamic and static skin deformation. It seems therefore justified to infer that in the human glabrous skin areas there are three types of intracutaneous mechanoreceptors with large diameter nerve fibres, one is rapidly adapting and two are slowly adapting, and in addition there is one rapidly adapting type of ending which is very sensitive to skin deformation although it is located in the subcutaneous tissues. This receptor is very likely a Pacinian corpuscle. It should be noted that it is not claimed that the four groups of units are necessarily homogeneous. A deeper analysis might reveal that there are several types of receptors within the classes of endings suggested on the basis of the present analysis.

In an analysis by Hagbarth *et al.* (1970) of mechanoreceptor activity from the same skin area in man only two types of receptors were found, rapidly and slowly adapting endings with distinct receptive field borders. This discrepancy compared with the present investigation might be due simply to the fact that a smaller unit sample (30 units) was collected in their study.

It is obvious that there was one definite difference between the present material and corresponding materials from the monkey. In man the slowly adapting receptors amounted to as much as three fourths of the total sample (75.4 %) whereas in the monkey the rapidly adapting receptors may constitute 80 % of the total sample and hence by far outnumber the slowly adapting ones (Lindblom 1965, Talbot *et al.* 1968). An important question is whether there was an over representation of slowly adapting units in the present study or not. This would be expected if the slowly adapting units in man had in general considerably larger nerve fibres than the fast

adapting ones as the recording technique selects large fibres. In the monkey, there is no difference in conduction velocities and hence in nerve fibre diameters between the slowly adapting units and the fast adapting ones (Talbot *et al.* 1968) and it appears unlikely that there would be in man. It seems therefore justified to conclude that there are, in fact, relatively more slowly adapting endings in man than in the monkey in these skin areas. The difference is remarkably large and it might be relevant to consider the possible implications of this finding. The higher proportion of slowly adapting receptors in man suggest that more accurate information of time invariant tactile stimuli is extracted by the human receptor population compared to that of the monkey. It is plausible that this implies an improvement of the hand as an organ for tactile exploration, which has occurred during the phylogenetic development.

Four of the slowly adapting mechanoreceptors encountered in the present study were spontaneously discharging and they were further characterized by their high sensitivity to stretching of the skin. The same properties have been described for the type II slowly adapting mechanoreceptor in the hairy skin of other mammals. In the present study it was demonstrated that the activities of these receptors could be increased as well as decreased by mechanical stimuli. This appeared in response to local indentations at different points, to extensions or flexions of the finger on which the receptor was located, or in response to stretching of the skin in different directions. These stimuli very likely gave rise to opposite mechanical effects on the structures at which the endings were anchored, one type caused an elongation and the other one a shortening of these structures. It is tempting to speculate about what could be the functional role of the two types of slowly adapting receptors as described in the present study. It is obvious that they supply totally different types of inputs to the central nervous system. The endings which have restricted receptive fields are activated exclusively when local indentations occur and the profile of activities in the population of afferent fibres must be very closely related in time course and spatial extent to the local indentation. The spontaneously active units, on the other hand, supply a continuous discharge in the absence of local mechanical stimuli. This discharge might be modified in either direction by a number of different mechanical factors which could affect the amount of stretch of the skin. For instance, a joint movement would probably modify the activities of a large number of these units from a wide region. Even if the impulse frequency of a single unit might not be very much altered, the total change of the input from the whole population on receptors would be considerable. It is likely that this activity is significant for the perceptive appreciation of the amount of stretch in the skin. However, it is possible that the endings also serve more specialized functions, such as supplying a kind of information about joint positions, which information could be significant for motor control.

This investigation was initiated with the idea that a description of the basic properties of the mechanoreceptors would be an essential prelude to analyses of neurophysiological and psychophysical responses in the same experiments to mechani-

cal stimulation of the human glabrous skin It was shown in one test series of the present study that such analyses are feasible with the present recording method

This investigation was supported by grants from the Swedish Medical Research Council (Grant No B69 14X 2075 03A) and University of Umeå (Reservationsanslaget för främjande av ograduerade forskares vetenskapliga verksamhet) The technical assistance of Mr Goran West ling is gratefully acknowledged

References

ADRIAN E D and Y ZOTTERMAN The impulses produced by sensory nerve endings Part 3 Impulses set up by touch and pressure *J Physiol* (Lond) 1926 *61* 464—483

BROWN A G and A IGGO A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit *J Physiol* (Lond) 1967 *193* 707—733

BUCHTHAL, F and A ROSENFALCK Evoked action potentials and conduction velocity in human sensory nerves *Brain Res* 1966 *3* 1—122

BURGESS P R D PETIT and R M WARREN Receptor types in cat hairy skin supplied by myelinated fibres *J Neurophysiol* 1968 *31* 833—848

CHAMBERS M R and A IGGO Slowly adapting cutaneous mechanoreceptors *J Physiol* (Lond) 1967 *192* 26 P—27 P

HAGBARTH K E and A B VALLBO Pulse and respiratory grouping of sympathetic impulses in human muscle nerves *Acta physiol scand* 1968 a *74* 96—108

HAGBARTH, K E and A B VALLBO Discharge characteristics of human muscle afferents during stretch and contraction *Exp Neurol* 1968 b *22* 674—694

HAGBARTH K E and A B VALLBO Single unit recordings from muscle nerves in human subjects *Acta physiol scand* 1969 *76* 321—334

[illegible]

1963 *24* 175—180

IGGO A Relation of single receptor activity to parameters of stimuli In *Touch heat and pain* Eds A V S de Reuck and J Knight Ciba Foundation J & A Churchill Ltd London 1966 237—256

IGGO A Electrophysiological and histological studies of cutaneous mechanoreceptors In *The skin senses* Ed D R Kenshalo C C Thomas Springfield Springfield 1968 84—105

IGGO A Cutaneous thermoreceptors in primates and sub-primates *J Physiol* (Lond) 1969 *200* 403—430

IGGO A and A R MUIR The structure and function of a slowly adapting touch corpuscle in hairy skin *J Physiol* (Lond) 1969 *200* 763—796

L[illegible] in distal glabrous skin of the monkey *J Neuro*[illegible]

L[illegible] from vibration sensitive receptors in the monkey [illegible]

MOUNTCASTLE V B W H TALBOT and H H KORNHUBER The neural transformation of mechanical stimuli delivered to the monkey's hand In *Touch heat and pain* Eds A V S de Reuck and J Knight Ciba Foundation J & A Churchill Ltd London 1966 325—345

TALBOT W H J DARIAN SMITH H H KORNHUBER and V B MOUNTCASTLE The sense of flutter vibration comparison of the human capacity with response patterns of mechanoreceptive afferents from the monkey hand *J Neurophysiol* 1968 *31* 301—334

VALLBO A B Slowly adapting muscle receptors in man *Acta physiol scand* 1970 *78* 315—333

VALLBO A B and K E HAGBARTH Impulses recorded with micro-electrodes in human muscle nerves during stimulation of mechanoreceptors and voluntary contractions *Electroenceph clin Neurophysiol* 1967 *23* 392

VALLBO A B and K E HAGBARTH Activity from skin mechanoreceptors recorded percutaneously in awake human subjects *Exp Neurol* 1968 *21* 270—289

WERNER G and V B MOUNTCASTLE Neural activity in mechanoreceptive cutaneous afferents stimulus response relations Weber functions and information transmission *J Neur*[illegible] 1965 *28* 359—397

Acta physiol. scand. 1970. 80. 196—214

From the Department of Physiology, Institute of Biology, University of Umeå, Sweden

Projections to the Cerebral Cortex from Afferents of the Interosseous Nerves of the Cat

By

H. SILFVENIUS

Received 23 February 1970

Abstract

SILFVENIUS, H. *Projections to the cerebral cortex from afferents of the interosseous nerves of the cat.* Acta physiol. scand. 1970. 80. 196—214.

Evoked focal potentials from the contralateral fore- and hindlimb interosseous nerves (Int.f. and Int.h.) as well as from Pacinian afferents were recorded in sensorimotor cortex and in S II in cats anaesthetized with chloralose. Low threshold, short latency potentials from Int.f. were evoked in the lateral sigmoid gyrus, in the region of the post cruciate dimple, in the rostral bank of the lateral ansate sulcus, in the lower bank of the anterior suprasylvian sulcus and in S II. The shortest latencies varied between 5.0—6.0 msec. Mechanical stimulation of dissected clusters of Pacinian receptors in the interosseous region evoked cortical responses in the same areas with slightly longer latencies. Low threshold electrical stimulation of Int.h. afferents evoked in addition to the known projection to S II (McIntyre 1962) initially positive potentials in two loci of the postsigmoid gyrus, one on the dorsal, the other on the medial aspect of the hemisphere. The latencies in these loci were as short as those in S II, i.e. 9.5—10.0 msec. Zonal convergence between projections from interosseous and Group I muscle afferents was observed. The Int.f. path ascends in the dorsal column. A cortical potential was, however, evoked in the postsigmoid gyrus by electrical stimulation of high threshold Int.h. afferents after transection of the dorsal columns at cervical level.

It is now a well established fact that proprioceptive afferents from the fore- and hindlimbs project to the sensorimotor cortex via short latency paths. Knowing the characteristics of receptor types subserved by afferents in specific nerves, it has been possible to study the course of these paths as well as their termination in the cerebral cortex with graded electrical stimulation of defined types of afferent axons and with adequate stimulation of the receptors themselves. Thus evidence has been provided that afferents from both Golgi and Ruffini organs in joints reach the cerebral cortex (Gardner and Noer 1952, Skoglund 1956, Mountcastle 1957, Andersen et al. 1967, Korner and Landgren 1969). Great attention has been focused on the cortical projection from Group I muscle afferents (Oscarsson and Rosén 1963, Oscarsson, Rosén and Sulg 1966, Landgren, Silfvenius and Wolsk 1967a, Landgren and Silfvenius 1969). The cortical projection from Ia afferents has been proved both with electrical and adequate activation of afferents and some evidence has also been provided that

Ib afferents independently of the Ia path reach the cerebral cortex (Landgren and Silfvenius 1969) Four types of proprioceptors have been identified in the interosseous nerves (Hunt and McIntyre 1960, Silfvenius 1970) The receptors are Pacinian corpuscles, tap receptors, tension receptors and muscle spindles The Pacinian afferents of these nerves have been found to project to the cerebral cortex (Mountcastle, Covian and Harrison 1952, McIntyre 1962, McIntyre, Holman and Veale 1967)

The accumulated knowledge of the cortical distribution of projections from proprioceptive afferents shows that, a least with regard to focal potentials, there exists a spatial convergence between some of the above mentioned different types of proprioceptive afferents Since the study of Mountcastle, Covian and Harrison (1952), no detailed focal potential study has been performed of the cortical projection of the forelimb interosseous nerve In a recent study of the receptors of the forelimb interosseous nerve (Silfvenius 1970) a hypothesis was advanced that Pacinian receptors might contribute with information utilized in motor mechanisms Little is known about the segmental reflex effects of Pacinian afferents According to McIntyre and Proske (1968) they do not affect the monosynaptic reflex of flexor muscles

In the cerebral cortex the projections of the Group I muscle afferents are located in area 3a, as well as in the border zone of the motor cortex i e in area 3 If Pacinian afferents contribute information utilized in motor mechanisms a cortical convergence between Group I and Pacinian afferents might be expected The present investigation was undertaken to elucidate the degree of zonal convergence between these two types of afferents in the cerebral cortex

Methods

Anaesthesia and general arrangements

15 cats were used Anaesthesia was induced by vaporizing 2 % Halothan Hoechst) in a Fluotec apparatus into a 2 1 gas mixture of oxygen nitrous oxide The anaesthesia was maintained with chloralose 70 mg/kg i v and supplemented occasionally late in the experiment with doses of 0 1—0 3 ml 3 % Nembutal (Abbott) i v to prevent reflex movements Gallamonium triethiodide Flaxedil (May and Baker) was used when spinal lesions were made The animal was then artificially ventilated with 96 % O_2 and 4 % CO_2 Blood pressure was monitored The temperatures of the animal and of the exposure pools were kept at 35—37° C The animal was mounted in a stereotaxic apparatus

Preparations for electrical stimulation of afferent nerves

The following nerves of the right limbs were dissected and prepared for electrical stimulation

A Forelimb nerves

The interosseous branch of the median nerve Int f
The deep radial nerve DR
The nerve to the I—III heads of the flexor digitorum profundus muscle DM
The superficial radial nerve SR
The superficial median nerve SM

B Hindlimb nerves

Th[illegible]

In addition to the nerves mentioned above the following hindlimb nerves were cut, the nerves to the anterior biceps, and semimembranous muscles, the peroneal nerve, the posterior tibial nerve, the posterior knee joint nerve, the plantaris nerve, the popliteus nerve, the flexor digitorum longus nerve, except the Int h and other tibial nerves distal to the Int h

In some experiments clicks were produced by feeding a 100 μsec square pulse from the [illegible]

to the extensor side of the forelimb where a pool was constructed The medial wound was then sutured and sealed This method enabled recording of the afferent volleys distally on the nerve-trunks

In the hindlimb the afferent volley of the Int h was recorded from the tibial nerve, those of the other nerves from the sciatic nerve

The nerves were cut peripherally and mounted on bipolar, chlorided silver wire electrodes under mineral oil for graded electrical stimulation For all the nerve inputs, the electrical stimulation strengths and the threshold of the cortical potentials are given in multiples of the strength (T) required to evoke a threshold response in the afferent nerve A 100 μsec square pulse was used for stimulation

Mechanical stimulation of Pacinian receptors

In the forelimb mechanical stim [illegible]
the preparation The cluster of [illegible]
depth of the interosseous space [illegible]
axially thus exposing the recepto [illegible]
the flexor side The forelimb was turned so that the receptor cluster was directly accessible to mechanical stimulation applied in a vertical direction When mechanical stimulation was used all the nerves to the forelimb were cut except Int f and those to the shoulder The intact Int f could thus be activated both by electrical stimulation (just proximal to the receptor cluster) and by adequate stimulation of its Pacinian receptors The cut SR, SM, DR and DM were stimulated electrically

In the hindlimb Pacinian receptors were considered to be activated by mechanical stimulation applied to the calcaneus Stimulating electrodes were hooked on the intact Int h

Mechanical stimulation of Pacinian receptors was provided by an electrodynamic mechanical stimulator using the same technique as described recently (Silfvenius 1970) Single mechanical pulses of variable duration and amplitude were used The time-constant of the steeply rising pulse delivered to the electrodynamic stimulator was 1 4 msec

The cervical cord was exposed between C_5 and C_7 when spinal cord lesions were made

The rostral part of the left cerebral hemisphere was exposed The anterior and lateral borders of the exposed area included the presigmoid gyrus the lateral sigmoid , the ectosylvian and the anterior part of the sylvian gyri The posterior border went about 10 mm behind the ansate sulcus The medial border followed the sagittal sinus

When hindlimb projections were studied the pre- and postsigmoid gyri were exposed about 10 to 15 mm posteriorly from the cruciate sulcus both on the dorsal and medial aspects of the left hemisphere Access to the medial cortex was gained by performing a frontal lobectomy of the right hemisphere and by removing the falx Accumulation of cerebrospinal fluid between the midline dural tissue and the hemisphere was avoided by lifting the sagittal dural tissue slightly off the hemisphere No interference with cortical circulation was observed

After the completion of the exposures the dorsal and medial surfaces of the left hemisphere were photographed with mm scales placed on the cortex Photograps of 7—8 × magnification were used for cortical mapping

The expression contralateral refers to the cerebral hemisphere

Recording and mapping of cortical evoked potentials

The evoked cortical potentials were recorded from the surface of the cortex with a spring mounted silver ball electrode or from deeper layers of the cortex with a penetrating micropipette electrode filled with Na-citrate and carried by a micromanipulator in a stereotaxic instrument

The projection fields were traced on the photographs of the cortex The border of the field was defined as the point where the amplitude of the evoked potential had decreased to 30 % of maximum Several individual experiments were summarized on tracings made from the [illegible] and the sulci The individual [illegible] rn A representation of the [illegible] ed

The extents of the spinal lesions were checked on histological sections stained with Luxol fast blue (Kluver and Barrera 1953) Tracings of typical sections were made at 20 × magnification

scope and photographed

Results

The response evoked in the cerebral cortex of the cat by mechanical stimulation of Pacinian corpuscles in the interosseous region and by electrical stimulation of low threshold afferents in the interosseous nerves was investigated in the following gyri and sulci the lateral sigmoid gyrus, the posterior sigmoid gyrus on the lateral and medial aspects of the hemisphere, the hidden banks of the ansate sulcus the anterior suprasylvian and ectosylvian gyri *the hidden banks of the anterior suprasylvian* sulcus

The distribution of the cortical projections of interosseous and Pacinian afferents is shown in Fig 1 The maps of Fig 1 summarize the typical features found in the present series of experiments They show the locations of short latency initially positive focal potentials recorded from the cortical surface and of similar initially negative potentials recorded in hidden cortical banks The maps include only areas in which initially negative focal potentials were found at cortical depths corresponding to layers III—V The focal potentials were evoked by electrical stimulation of

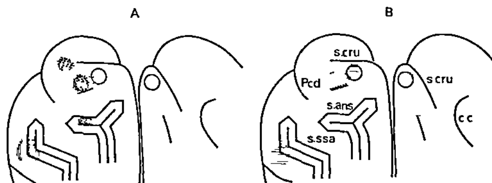


Fig 1 Diagram of cortical field distribution of focal potentials evoked by low threshold interosseous and Group I muscle afferents

A Forelimb pattern Int f projection areas vertical hatching Group I muscle afferent projection DR DM stippled Group I hindlimb projections encircled areas

B Hindlimb pattern Int h projection areas horizontal hatching Group I hindlimb and forelimb as in A

The dorsal aspect of the hemisphere is shown to the left in each diagram the medial aspect to the right S cru —cruciate sulcus Pcd—post cruciate dimple S ans =ansate sulcus S.ssa.= anterior suprasylvian sulcus c c —corpu

The hidden banks of S ans and S ssa

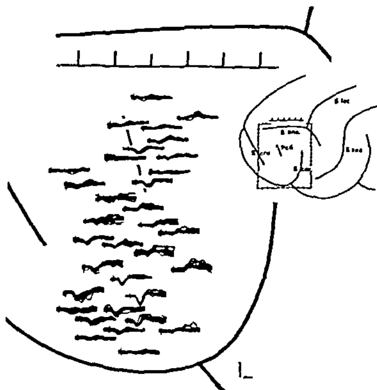


Fig 2 Cortical distribution of potentials evoked by low threshold electrical stimulation of contralateral Int f The area bordered by dotted lines in the inset diagram to the right corresponds to the enlargement Two maxima of initially positive surface potentials are shown One is located near the Ped the other in the lateral sigmoid gyrus The artifact of each response indicates the recording position mm scales in both figures Abbreviations as in Fig 1 S lat = lateral sulcus S cor = coronal sulcus Vertical bar 400 μV Horizontal bar 10 msec Positivity downward in this and subsequent records

the interosseous nerve of the contralateral forelimb Int f and of the contralateral hindlimb Int h The stimulus strengths used for mapping did not exceed twice threshold value T of the afferent volley as recorded from the nervetrunks

The projections of Int f (Fig 1 A vertical hatching) and Int h (Fig 1 B horizontal hatching) are related to the projection fields of the Group I muscle afferents of the contralateral forelimb (stippling) and to those of the Group I muscle afferents of the contralateral hindlimb (encircled areas) The different Int f and Int h projections will be described in some detail below

I Int f projections to the lateral and the posterior sigmoid gyri

The sigmoid projections of the contralateral Int f were located in the region of the postcruciate dimple Ped and in the lateral sigmoid gyrus near the lateral end of the cruciate sulcus These two projection fields were often confluent but in 30 per cent of the investigated animals two loci with evoked potentials of maximal amplitude were observed The two loci were separated by a nonresponding zone or by an area in

which responses of lower amplitudes were recorded Such a separation is illustrated in Fig 2

The Pcd locus was 1—3 mm in diameter and located near the dimple The locus of the lateral sigmoid gyrus was 0 5—2 0 mm in diameter, and the point where the potential had a minimal latency and a maximal amplitude was generally found 1—3 mm lateral to the end of the cruciate sulcus The rostro-lateral border of the lateral sigmoid Int f locus was always distinct

The Int f projections to Pcd overlapped in general with the rostrolateral part of the projection field of the Group I muscle afferents of the contralateral forelimb (Oscarsson and Rosen 1963) (*cf* Fig 1 A) A separate Group I projection area in the lateral sigmoid gyrus was described by Silfvenius (1968) and the Int f locus in this region overlapped with the Group I forelimb projection It also overlapped with the rostral projection area of the low threshold skin afferents of the superficial radial nerve SR (*cf* Oscarsson and Rosen 1966) Neither in the Pcd region nor in the lateral sigmoid gyrus were the fields of forelimb Group I and Int f afferents identical in location and extent The Int f projections to the lateral sigmoid gyrus fall within the cytoarchitectonic area 4 of Hassler and Muhs Clement (1964) *i e* the motor cortex The Pcd locus is located in the border zone of the motor cortex corresponding to area 3

The threshold of the potential evoked by Int f near Pcd was 0 9 T (mean of 9 expts range 0 6—1 2 T) In the lateral sigmoid gyrus the threshold was 1 0 T (mean of 8 expts range 0 6—1 1 T) The cortical responses thus appeared simultaneously with or even before the first sign of afferent volley in the median nerve The growth in amplitude of the cortical evoked potential with increasing strength of electrical stimulation of Int f is shown in Fig 3 A The amplitudes of the potentials evoked near Pcd (▽) in the lateral sigmoid gyrus (▼) and in the anterior ectosylvian gyrus (second somatosensory projection area SII (○) are plotted against stimulus strengths The increa e in amplitude of the cortical evoked potential may be compared with that of the afferent volley (*cf* Fig 3 B) There is a steep increase in amplitude of the afferent volley and of the cortical potentials when the strength of stimulation is increased from 1 0 to 1 5 T With further increase in stimulation intensity there is a slow gradual increase in amplitude of the afferent volley between 2 T and 10 T The cortical potentials however do not increase in amplitude above 2 T Maximal amplitude of the potential evoked by Int f in the Pcd region was reached at 1 2—2 0 T with a mean of 1 6 T in 8 experiments In the lateral sigmoid gyrus the amplitude maximum of the Int f potential was reached at strengths between 1 2 T and 1 8 T (mean 1 5 T in 7 experiments) It is thus obvious that the Int f projection to the sigmoid gyrus are due to low threshold interosseous afferents

The amplitude of the Int f potential evoked in the Pcd region was generally about 50 per cent of the Int f potentials evoked in the SII projection Area (*cf* Fig 3 A) In the lateral sigmoid gyrus the amplitude of the Int f potential varied being at times of similar magnitude as that recorded in SII but also lower maximal amplitudes were observed

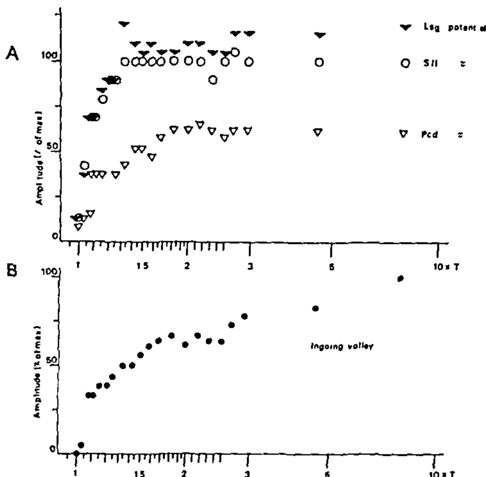


Fig 3 A Correlation between stimulus strength and cortical responses evoked by Int f in the lateral sigmoid gyrus (▼) in Pcd (▽) and in S II (⊙) The amplitudes of the initial positive components of the cortical surfase potentials were measured
B Correlation between stimulus strength and amplitude of the ingoing Int f volley (●)

The latencies of the cortical potentials evoked by Int f are given in Table I The shortest latency observed in the Pcd locus and in the lateral sigmoid gyrus was 6 msec, which is 1–2 msec longer than the shortest latencies of the potentials evoked in these regions by forelimb Group I muscle afferents and forelimb low threshold skin afferents In the present dissection the peripheral conduction distances differed in that the SR and DR nerves were about 20 mm shorter than the other ones stimulated

II Int f projection to the rostral bank of the lateral ansate sulcus

A focal potential was evoked in the rostral bank of the lateral ansate sulcus by electrical stimulation of the contralateral Int f This region was explored with penetrating microelectrode tracks in 8 animals In 6 of them the Int f response appeared with stimulus strength just threshold for the afferent volley and the cortical potential

TABLE I Minimum latencies of low threshold potentials recorded in different cortical areas and

Forelimb afferents	Cortical region	
	lat sigm gyrus	Pcd region
Int f	6 0—9 0, 7 6±0 8 (11)	6 0—9 5 7 3±0 3 (14)
DM	5.2—9 5 7 1±0 4 (9)	5 5—7 0 6 2±0 2 (13)
SM	5 0—9 5, 7 6±0 7 (9)	5 0—11 0, 7 3±0 5 (13)
SR	4 5—8 0, 5 8±0 4 (9)	5 0—8 0 6 1±0 3 (13)
	5 5—10 0, 6 9±0 5 (10)	4 2—7 0, 5 3±0.2 (13)

Hindlimb afferents	Dorsal hindlimb locus in postsigmoid gyrus
Int h	9 5—17.5, 12 1±0 9 (8)
G	11 5 (2)
PBSt	11 1 (2)
Su	9 5—13 5, 10 7±0 7 (6)

Range, means and standard deviations from mean are given Numbers in brackets indicate number corded either on the surface or in the depth of the cortex

reached maximal amplitude below a strength of 2 T In 1 expt Int f responses were evoked only by stimulation above 2 5 T, and in 2 expts no responses to stimulation of Int f were found presumably because the cortical surface vessels prevented a full exploration of the rostral bank

The Int f projection field was confined to the rostral bank of the lateral ansate sulcus It was found 0 5—2 mm below the surface of the adjacent gyri with a maximal amplitude of the evoked potential at a depth of 1 0—1 5 mm The mediolateral extent was 1—2 mm The Int f field overlapped with the projection of the Group I muscle afferents from the contralateral forelimb a projection recently described (Silfvenius 1968) These two fields were however not coextensive Responses to electrical stimulation of low threshold cutaneous afferents from the contralateral forelimb were also evoked within the Int f projection area No Int f responses were recorded on the cortical surface of the nearby caudal projection area of the superficial radial nerve C—SR described by Oscarsson and Rosen (1963 1966) nor were any responses observed in the corresponding part of the projection field of the median cutaneous afferents, located somewhat medially to C—SR on the postsigmoid gyrus The Int f projection hidden in the rostral bank of the lateral ansate sulcus is thus located at the caudal border of the forelimb field in the first somatosensory projection area (SI)

The shortest latencies observed of the Int f responses recorded in the ansate region were 5 0 msec (*cf* Table I) a value similar to the latencies of the potentials evoked in this region by Group I muscle and low threshold cutaneous afferents

III Int f projections to the anterior ectosylvian gyrus SII and to the anterior suprasylvian sulcus and gyrus

Mountcastle Covian and Harrison (1952) demonstrated that light mechanical stimulation of an isolated limb *i e* a limb deprived of exteroceptive input evoked cortical potentials which were mediated via the interosseous nerves These focal po

evoked by electrical stimulation of contralateral afferent nerves

Group I locus in rostral ansate bank	Cortical region Group I locus in lower bank of ass	SII forelimb area
5 0—7 0, 6 4±0 4 (5)	5 0—8 5, 6 5±0 4 (7)	5 5—9 5, 7 6±0 4 (11)
5 6—8 5, 6 6±0 4 (7)	6 0—7 0, 6 4±0 2 (6)	—
6 0—9 5, 7 3±0 5 (8)	5 5—8 0, 6 4±0 4 (7)	5 0—9 5, 6 5±0 4 (10)
5 4—8 0, 6 3±0 4 (7)	4 7—6 0, 5 5±0 2 (6)	4 0—6 5, 5 3±0 3 (9)
4 7—7 0, 5 3±0 3 (7)	4 8—6 0, 5 4±0 2 (6)	
Medial hindlimb locus *in postsigmoid gyrus*		SII hindlimb area
10 0—18 0 12 2±1 3 (6)		9 5—14 0, 12 0±0 4 (10)
9 0—11 0 10 0 (3)		—
11 3 (2)		—
9 0—10 0, 9 6±0 3 (4)		9 0—13 5 11 0±0 5 (10)

of animals from which the observations were obtained The shortest latencies are given being re

tentials were of large amplitude in SII and somewhat smaller in SI McIntyre (1962) and Norrsell and Wolpow (1966) also showed that low threshold electrical stimulation of Int h evoked focal potentials in these cortical areas

The present investigation confirms and extends the results of the earlier studies The contralateral Int f evoked responses of larger amplitude in SII than in any other cortical Int f projection area The point where the Int f potential showed a maximal amplitude was located in the ectosylvian gyrus near the lower bend of the anterior suprasylvian sulcus The rostro caudal extent of the projection area varied between 1 5 and 8 mm with a mean of 3 5 mm As shown in Fig 1 A the area covered mainly the caudal half of the part of the ectosylvian gyrus that borders the anterior suprasylvian sulcus In the lateral direction the field reached about halfway between the anterior supra and ectosylvian sulci The Int f projection area was always smaller than that of the low threshold cutaneous afferents from the contralateral forelimb (SR SM)

When the hidden cortical banks of the anterior suprasylvian sulcus were investigated with penetrating microelectrode tracks it was found that the Int f *projection area extended down into the cortex of the lower* bank of this sulcus In this bank Int f evoked an initially positive focal potential near the hidden surface of the bank About 0 5 mm below this level *i e* in layer III—V the potential changed into an initially negative one It was maximal in amplitude in the lower bank near the lower bend of the anterior suprasylvian sulcus thus near the maximum found on the surface of the anterior ectosylvian gyrus The rostro caudal extent along the lower bank of the sulcus varied from 3—6 mm The rostral border was located somewhat caudal to the rostral end of the anterior suprasylvian sulcus and the caudal border extended *somewhat caudally to the lower bend of this sulcus* The Int f projection generally extended to the depth of the sulcus Its mediolateral extent varied between 1 and 4 mm

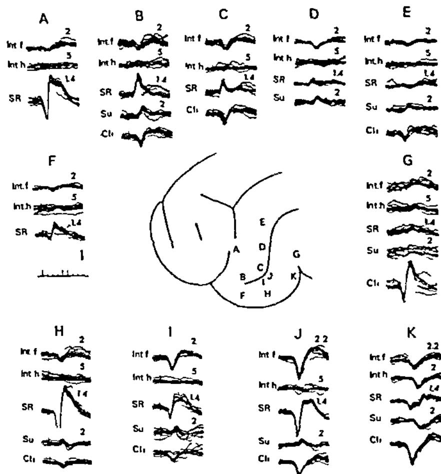


Fig 4 Convergence in anterior suprasylvian and ectosylvian gyri of low threshold cortical focal potentials evoked by activation of different fore and hindlimb afferents as well as by auditory clicks The superimposed records were obtained from regions indicated with capital letters Numbers at end of records indicate the used strength of stimulation expressed in multiples of T Timer 5 msec Voltage bar 100 μV

Projections from Group I forelimb muscle afferents from auditory and vestibular afferents to the lower bank of the anterior suprasylvian sulcus are previously described by Landgren Silfvenius and Wolsk (1967a and b) The Int f field of the lower bank overlaps these projections (*cf* Fig 1 A)

Electrical stimulation of Int f also evoked initially positive surface potentials in the cortex of the anterior suprasylvian gyrus (*cf* Fig 4 B—E) and in the upper bank of the anterior suprasylvian sulcus These responses however differ from those evoked in the lower bank of the sulcus and in the ectosylvian gyrus A shift to an initially negative focal potential was never observed in microelectrode records from layers III–

—V of the anterior suprasylvian gyrus nor in the upper bank of the anterior suprasylvian sulcus

The cortical potentials recorded in the ectosylvian gyrus and in the suprasylvian sulcus were evoked by low threshold Int f afferents The threshold in the gyrus was 1 T *(mean of 9 expts range 0 7—1 1 T) In the lower bank of the sulcus a similar* threshold was observed (1 T mean of 5 expts range 0 6—1 3 T) In both recording sites the evoked potentials reached maximal amplitudes at 1 5 T

The latencies of the potentials were short both in the gyrus and in the lower bank As shown in Table I the shortest Int f latency observed was 5 0 msec in the lower bank and 5 5 msec in the ectosylvian gyrus These latencies are similar to those of the potentials evoked in the region by Group I forelimb muscle afferents The Int f latencies increased by 1– 3 msec when recorded in the caudal part of the projection area which overlapped with the auditory and the vestibular projections *i e* with the composite sensory area (*cf* Landgren Silfvenius and Wolsk 1967 b) A similar prolongation of latency was also observed when Int f responses from the point of maximal amplitude in the anterior ectosylvian gyrus were compared with those recorded in area SII A of Carreras and Andersson (1963) The Int f potentials of the upper bank of the anterior suprasylvian sulcus and of the anterior suprasylvian gyrus areas not showing reversals to negative focal potentials in layers III—V also had slightly longer latencies ranging from 7 5 to 14 5 msec

IV Int h projections to the anterior ectosylvian gyrus and to the hindlimb areas of the posterior sigmoid gyrus

Previous observations of low threshold Int h projections to the anterior ectosylvian gyrus were confirmed (McIntyre 1962 Norrsell and Wolpow 1966) In addition the overlap between the Int f and Int h projection areas in the anterior ectosylvian gyrus was studied The overlap is indicated by the records obtained from point K in Fig 4 where an Int f response with an amplitude about 50 per cent of the maximum was recorded together with a response evoked by Int h A comparison between Fig 1 A and B also shows this overlap

In the present series of experiments Int f projections were found to overlap with all the projection areas of the forelimb Group I muscle afferents It was therefore considered of interest to investigate the possibility of a similar overlap between Int h and hindlimb Group I muscle afferents The last mentioned afferents project to two loci in the posterior sigmoid gyrus as described by Landgren and Silfvenius (1969) One of the hindlimb Group I loci was found on the dorsal and the other on the medial surface of the hemisphere Int h projections to the dorsal aspect of the posterior sigmoid gyrus are described by McIntyre (1962) and confirmed by Norrsell and Wolpow (1966) The medial aspect of the hemisphere was not investigated in their studies

The projection areas of the hindlimb Group I muscle afferents were mapped together with the projection areas of low threshold (= 1 5 T) Int h afferents and with hindlimb low threshold cutaneous afferents It was found that Int h afferents pro-

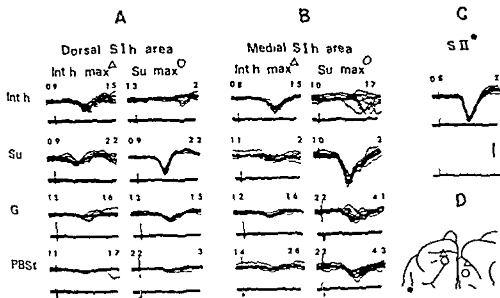


Fig 5 Comparison between low threshold cortical local potentials evoked by Int h, muscle and skin afferents. The upper record in each pair shows the superimposed cortical potentials, the lower record shows the ingoing volley

C Upper record shows the cortical potential evoked in the [illegible] (●), electrical stimulation of Int h Lower record shows ingoing volley

D Inset diagram showing the recording positions for the maximal points of Int h and Su projections The numbers to the left of each record of cortical focal potentials indicate cortical thresholds those to the right the stimulation strengths used

Timer 5 msec Voltage bar 100 μV

jected to both Group I loci The Group I and the Int h projections were however not identical, i e the locations of their maximal points and the course of their border lines differed (cf Fig 1 B) The maximum points of the Int h projections were located somewhat rostrally to the corresponding points of the Group I projections The extent of the Int h projections to the postsigmoid gyrus was about 1 mm or less

There was generally a better agreement between the distribution of Int h and Group I projections than between Int h and cutaneous projections As previously observed by Landgren and Silfvenius (1969) low threshold sural afferents have two maximum points (○ Fig 5 D) The potentials evoked by Int h in the sural maximum points showed low amplitudes and long latencies Likewise the sural responses were of low amplitude in the Int h maxima Fig 5 also demonstrates that the amplitudes of the potentials evoked by Int h in the posterior sigmoid gyrus were about 50 per cent of those observed in the SII projection area (Fig 5 A—C)

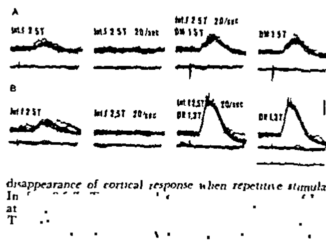


Fig 6 Records of low threshold *cortical focal potentials obtained in the Group I forelimb locus of* the lower bank of the anterior suprasylvian sulcus and evoked by separate and combined stimulation of Int f and Group I forelimb muscle afferents. Upper records in A and B cortical potentials lower records ingoing volleys

A First record, superimposed records evoked by a single shock at 2 5 T to Int f Second record *disappearance of cortical response when repetitive stimulation at 20/sec was delivered to the* In [illegible] d of DM 1/se at [illegible] to DM at 1.5 T [illegible]

The thresholds of the responses evoked by Int h in the posterior sigmoid gyrus were 1 T The amplitudes increased rapidly when the stimulus strength was raised *from 1 0 to 1 5 T Above 2 T there occurred a gradual increase in amplitude of the* evoked potentials indicating a response to high threshold Int h afferents In the SII hindlimb projection area no additional response was seen when high threshold af*ferents of Int h were activated*

The latencies of the potentials evoked in the postsigmoid gyrus by low threshold Int.h afferents were short and comparable with those of the potentials evoked by the low threshold cutaneous afferents and the Group I muscle afferents (*cf Table I*)

I *Convergence of afferent inflow to the Int f projection areas*

The overlap between the cortical projection areas of the interosseous the Group I muscle and the cutaneous afferents has been mentioned above It was however emphasized that the maximum points and the extents of the areas differed The findings indicate a zonal convergence between these three groups of low threshold afferents The convergence of the afferent inflow was not studied on a neuronal level in the present investigation Some observations were however made concerning the interdependency of the afferent paths

The records of Fig 6 were obtained from the lower bank of the anterior suprasylvian sulcus The recording site was the forelimb Group I locus (Landgren Silfvenius and Wolsk 1967 a) Large focal potentials were evoked by Group I muscle afferents (Fig 6 DM and DR) A response was also evoked by Int f The Int f path was then activated by repetitive stimulation at 20 shocks per sec which blocked the transmission. A simultaneous stimulation of Group I afferents in the muscle nerves DM and DR evoked a large cortical response which did not increase in amplitude when the stimulation of Int f was stopped The result demonstrates that the Int f and the Group I paths are largely independent of one another

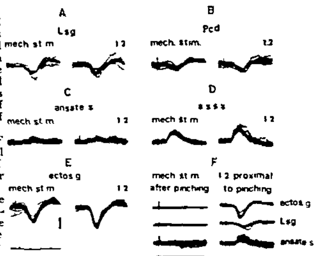


Fig 7 Comparison of cortical potentials evoked by mechanical stimulation of Pacinian corpuscles (to the left) and by electrical stimulation of Int f at a strength of 1 2 T (to the right) The cortical potentials were recorded from the lateral sigmoid gyrus (A) Pcd (B) rostral bank of ansate sulcus (C), lower bank of anterior suprasylvian sulcus (D) and ectosylvian gyrus (E) F shows the response to mechanical stimulation after pinching of Int f just proximal to the receptor cluster (to the left) and to electrical stimulation proximal to the pinching (to the right) Mechanical stimulation with single pulses 0 18 mm in amplitude and 1 msec duration was delivered to the cluster of Pacinian corpuscles of the forelimb interosseous region Pulse artifact shown in lowest left record under F Timer 5 msec Voltage bar 200 μV

1 I Identification of Int f receptors

There are four types of identified mechanoreceptors with rapidly conducting afferent axons travelling in Int f (Silfvenius 1970) The afferents from these mechanoreceptors might accordingly have contributed to the cortical potentials evoked by low threshold electrical stimulation of Int f In order to establish the contribution of Pacinian afferents to the cortical potentials mechanical single pulse stimulation of the cluster of Pacinian corpuscles in the forelimb interosseous space was performed as described in Methods The distribution of the cortical potentials evoked by mechanical and electrical stimulation was compared in two experiments All the Int f projections described above were investigated It was found that selective activation of Pacinian afferents evoked cortical potentials with the same distribution as did low threshold electrical stimulation of Int f The findings are illustrated in Fig 7 (A—E) which also shows that mainly Pacinian afferents are responsible for the cortical potentials evoked by the low threshold Int f afferents The amplitude of the cortical potentials evoked by mechanical stimulation was 60 to 80 per cent of that of the potentials evoked by electrical stimulation (1 2 T) Fig 7 F finally shows that the response to mechanical stimulation was due to Int.f afferents only and not to activation of remotely located Pacinian receptors

McIntyre Holman and Veale (1967) have shown that mechanical stimulation of single Pacinian receptors of the hindlimb interosseous region evokes cortical potentials in the SII projection area In the present investigation it was observed that a single mechanical pulse delivered to the calcaneus and presumably stimulating Pacinian corpuscles evoked responses in the hindlimb areas of the postsigmoid gyrus as well as in the S II

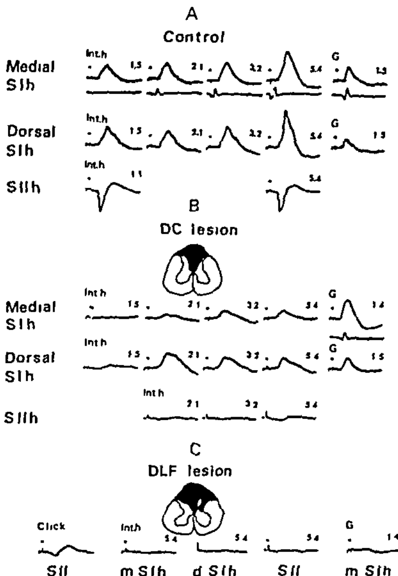


Fig 8 Records of computed averages (50 sweeps) of cortical focal potentials evoked by the contralateral Int h and Group I afferents of the gastrocnemius muscle G A before B after cervical (C_4) transection of the dorsal columns and C after an additional ipsilateral (C_5) transection of the dorsolateral fascicle The diagrams give the extents of the lesions

The cortical potentials were recorded from layers III—V of the medial (A and B upper row, medial (m) S I h) and dorsal (A and B middle row dorsal (d) S I h) postsigmoid hindlimb areas *Surface potentials from S II are shown in the lower rows* (A and B) Ingoing volleys recorded with fast sweep speeds are shown under some of the records Figures to the right give strength of stimulation Calibration pulse of cortical records amplitude 100 μV, duration 2 msec

I II The spinal course of the ascending interosseous paths

Afferents from the forelimb receptors responding to high frequency vibration have been shown to ascend in the dorsal column system of the spinal cord (Uddenberg 1968) The location of the ascending Int f path in the spinal cord was investigated by performing spinal transections at C_4 level It was found that the Int f path ascends in the dorsal columns (DC) The cortical responses evoked in all Int f loci by low threshold electrical stimulation were abolished after transections of DC Confirming observations were made in experiments in which mechanical stimulation of Pacinian corpuscles was used thus proving that the response originated from these receptors The spinal course of the high threshold Int f afferents was not investigated

McIntyre (1962) and Norrsell and Wolpow (1966) have studied the spinal course of the Int h path They found that the low threshold component of the cortical Int h response disappeared after transection of DC This was confirmed in the present study Fig 8 shows that the potentials evoked by electrical stimulation of Int h at 1 5 T disappeared in the two loci of the postsigmoid gyrus as well as in the ectosylvian gyrus When the stimulus strength was increased above 2 T no high threshold response was evoked in the SII hindlimb projection area In the Int h loci of the postsigmoid gyrus however a high threshold component of the cortical potential remained after DC transection (*cf* Fig 8 B) The latencies of these responses were 1—2 msec longer than those evoked by the low threshold Int h afferents The potentials evoked in the postsigmoid gyrus by the hindlimb Group I muscle afferents remained unaltered after the DC interruption An additional transection of the dorsolateral fascicle (DLF) ipsilateral to the afferent nerves abolished both the response to the high threshold Int h and to the Group I muscle afferents After the DLF lesion auditory stimuli still evoked a response in the S II A area of Carreras and Andersson (1963)

Discussion

There is a considerable number of Pacinian afferents in the interosseous nerve but graded electrical stimulation of these nerves does not selectively activate Pacinian afferents Rapidly conducting afferents from four types of different mechanoreceptors have been identified in the interosseous nerves The receptors are Pacinian corpuscles tap receptors tension receptors and muscle spindles (Hunt and McIntyre 1960 *Silfvenius 1970*) *All of them contribute to the group of afferents with axon diame*ters between 12 and 19 μ and are discharged by low threshold electrical stimulation For this reason the cortical projection pattern determined by electrical stimulation of the interosseous nerve cannot be interpreted as Pacinian projections only In the present series however it was demonstrated that direct mechanical stimulation of Pacinian corpuscles evoked cortical potentials with similar amplitudes and with identical distribution as did electrical stimulation of the low threshold ($<$1 5 T afferents in the interosseous nerves It cannot be excluded that some nearby mechanoreceptors other than the Pacinian ones could have been excited by the

mechanical pulse but the cluster of Pacinian corpuscles near the stimulating probe must have contributed predominantly to the afferent volley It is therefore reasonable to accept the low threshold interosseous projections described in this paper as representative of Pacinian projections to the cerebral cortex

The Pacinian afferents of the Int f project to area 4 , i e to the part of the motor cortex characterized by large Betz cells van Crevel and Verhaart (1963) demonstrated that at least 80 per cent of the large corticospinal fibres (>6 μ) originate in the pericruciate cortex which includes area 4 , *The cortical origin of the most direct and fast conducting efferent path to the segmental motoneurones is thus located in area 4* In addition to the Pacinian input this area receives projections from low threshold cutaneous afferents of the forelimb (Oscarsson and Rosen 1963 1966) and from large muscle spindle afferents in the contralateral forelimb (Silfvenius 1968) Exteroceptive and proprioceptive impulses discharge the pyramidal tract cells of area 4 as shown by Asanuma Stoney and Abzug (1968) The Pacinian projections to area 4 are presumably part of the proprioceptive and exteroceptive afferent input which affects the corticospinal output Synaptic connection between the Pacinian projections and identified single pyramidal tract cells have so far not been demonstrated It also remains to be established whether the observed zonal convergence between the Pacinian and the Group I projections is due to a convergence on single cortical neurones The available knowledge nevertheless suggests that the Pacinian projections to area 4 , are utilized in motor control mechanisms

The short latency of the response evoked in the motor cortex by the Pacinian afferents indicates that the ascending path has a small number of synaptic relays The first of these synapses is probably located in the main cuneate nucleus as suggested by the observations of Amassian and de Vito (1957) The findings of Mountcastle Covian and Harrison (1952) suggest that the ventrobasal complex of the thalamus is the site of the second synapse The latencies of the Pacinian responses evoked in the lateral sigmoid gyrus and in Pcd are however long enough to allow still another synapse in the path (*cf* Table I) These latencies are in fact 1 msec longer than those of the Pacinian responses evoked in the ansate and anterior suprasylvian sulci The possible existence and the location of an extra synapse remains to be demonstrated It is also necessary to investigate whether the thalamo-cortical link of the path includes different neurones to the different cortical projection areas or one neurone with axonal branches to several cortical loci (*cf* Andersen Andersson and Landgren 1966 Andersson Landgren and Wolsk 1966 Rowe and Sessle 1968 Manson 1969)

Pacinian afferents from the contralateral forelimb and hindlimb project to area 3 i e to the border zone of area 4 / This border zone also receives input from Group I muscle afferents low threshold joint afferents and low threshold skin afferents (Oscarsson and Rosen 1963 1966 Landgren and Silfvenius 1969 Korner and Landgren 1969) *A zonal convergence between these projections* was thus observed A convergence between the Group I and the cutaneous projections on single cortical cells was demonstrated by Oscarsson Rosen and Sulg (1966) and by Swett and

Bourassa (1967) Similar evidence of convergence on identified cortical cells is not yet available for the Pacinian and joint projections

According to Jones and Powell (1968) reciprocal corticocortical connexions exist between areas 3 and 4 Both areas contribute axons to the pyramidal tract (*cf* van Crevel and Verhaart 1963) The functions of the two regions therefore seem to be related The observation that Pacinian afferents evoke responses in both areas with the same latency does not support the alternative that the Pacinian input is relayed to area 4 via area 3 Further experiments are required to elucidate the functional significance of the correlation between the two cortical areas

In addition to the projections to the motor cortex and its border zone the Pacinian afferents project to separate areas related to the primary somato-sensory projections *i e* to SI and SII The SI projection is not found on the surface of the postsigmoid gyrus, *i e* rostral to the ansate sulcus where the projections of the low threshold cutaneous afferents from the contralateral forelimb are located The Int f area is hidden in the rostral bank of the ansate sulcus and overlaps the most caudal part of the cutaneous projections Again a zonal convergence was observed in the ansate sulcus between the Pacinian and the Group I muscle afferent projections from the forelimb

The Pacinian projections to SII evoke a potential of conspicuous amplitude The field is located more caudally than that of the cutaneous projections and it overlaps the composite sensory area, which is located in the anterior ectosylvian gyrus and the anterior suprasylvian sulcus, thus between SI SII and the auditory projections A zonal convergence between Pacinian and Group I muscle afferents was also observed in the Group I locus of the anterior suprasylvian sulcus

The finding that the Pacinian afferents ascend in the dorsal columns is in agreement with previous observations (McIntyre 1962 Uddenberg 1968 Burgess and Clark 1969) It is however interesting to note that a component of the response evoked by Int h afferents in the postsigmoid gyrus remained after transection of the dorsal columns This response had a threshold between 1 5 and 2 T and is thus not due to Pacinian afferents The receptors from which this projection originates are unknown They may however, in part represent tension receptors with Group Ib afferents travelling in the dorsolateral fascicle togethe with the Group Ia muscle afferents from the hindlimb A component of higher threshold was not observed in SII where Int f as well as Int h responses disappeared after transection of the dorsal columns

I gratefully acknowledge the advice and criticism of Professor S Landgren Miss Ewa Lignell gave technical assistance This investigation was partially supported by a grant from the Medical Faculty University of Umeå and partially by the Swedish Medical Research Council (Project No B70-14X-45-06)

References

ASTASSIAN V E and J L DE VITO La transmission dans le noyau de Burdach. *Colloq int Centre Natl Rech Sci* (Paris) 1957 *67* 353—393

ANDERSEN P S ANDERSSON and S LANDGREN Some properties of thalamic relay cells in the spinocervico-lemniscal path *Acta physiol scand* 1966 *68* 72—83

Andersen H T, L Korner S Landgren and H Silfvenius Fibre components and cortical projections of the elbow joint nerve in the cat *Acta physiol scand* 1967 *69* 373—382
Andersson S A S Landgren and D Wolsk The thalamic relay and cortical projection of Group I muscle afferents from the forelimb of the cat *J Physiol* (Lond) 1966 *183* 576—591
Asanuma H S D Stoney and C Abzug Relationship between afferent input and motor outflow in cat motorsensory cortex *J Neurophysiol* 1968 *31* 670—681
Burgess P R and F J Clark Dorsal column projection of fibres from the cat knee joint *J Physiol* (Lond) 1969 *203* 301—315
Carreras M and S A Andersson Functional properties of neurones of the anterior ectosylvian gyrus of cat *J Neurophysiol* 1963 *26* 100—126
van Crevel H and W J C Verhaart The "exact" origin of the pyramidal tract *J Anat* (Lond) 1963 *94* 495 515
Gardner E and R Noer Projection of afferent fibres from muscles and joints to the cerebral cortex of the cat *Amer J Physiol* 1952 *97* 199—221
Hassler R and K Muhs Clement Architektonischer Aufbau des sensorimotorischen und parietalen Cortex der Katze *J Hirnforsch* 1964 *6* 377—420
Hunt C C and A K McIntyre Characteristics of responses from receptors from the flexor longus digitorum muscle and the adjoining interosseous region of the cat *J Physiol* (Lond) 1960 *153* 74—87
Jones E G and T P S Powell The ipsilateral cortical connexions of the somatic sensory areas in the cat *Brain Res* 1968 *9* 71—94
Kluver H and E Barrera A method for the combined staining of cells and fibres in the nervous system *J Neuropath exp Neurol* 1953 *12* 400—403
Korner L and S Landgren Projections of low threshold joint afferents to the cerebral cortex of the cat *Acta physiol scand* 1969 *76* 5A—7A
Landgren S and H Silfvenius Projection to cerebral cortex of Group I muscle afferents from the cat s hindlimb *J Physiol* (Lond) 1969 *200* 353—372
Landgren S H Silfvenius and D Wolsk Somato sensory paths to the second cortical projection area of the Group I muscle afferents *J Physiol* (Lond) 1967 a *191* 543—559
Landgren S H Silfvenius and D Wolsk Vestibular cochlear and trigeminal projections to the cortex in the anterior suprasylvian sulcus of the ca *J Physiol* (Lond) 1967 b *191* 561 573
Manson J The somatosensory cortical projection of single nerve cells in the thalamus of the cat *Brain Res* 1969 *12* 489—492
McIntyre A K Cortical projections of impulses in the interosseous nerve of the cat s hind limb *J Physiol* (Lond) 1962 *163* 46—60
McIntyre A K and L Proske Reflex potency of cutaneous afferent fibres *Aust J exp Biol med Sci* 1968 *46* 19
McIntyre A K M E Holman and J L Veale Cortical responses to impulses from single Pacinian corpuscles in the cat s hind limb *Exp Brain Res* 1967 *4* 243—255
Mountcastle V B Modality and topographic properties of single neurones of cat s somatic sensory cortex *J Neurophysiol* 1957 *20* 408—434
Mountcastle V B M R Covian and C R Harrison The central representation of some forms of deep sensibility *Res Publ Ass nerv ment Dis* 1952 *30* 339—370
Norrsell U and E R Wolpow An evoked potential study of different pathways from the hindlimb to the somatosensory areas in the cat *Acta physiol scand* 1966 *66* 19—33
Oscarsson O and I Rosen Projections to cerebral cortex of large muscle spindle afferents in forelimb nerves of the cat *J Physiol* (Lond) 1963 *169* 924—945
Oscarsson O and I Rosen *Short latency projections to the cat s cerebral cortex from skin* and muscle afferents in the contralateral forelimb *J Physiol* (Lond) 1966 *182* 164—184
Oscarsson O I Rosen and I Sulg Organization of neurones in the cat cerebral cortex that are influenced from Group I muscle afferents *J Physiol* (Lond) 1966 *183* 189—210
Rowe M J and B J Sessle Somatic afferent input to posterior thalamic neurones and the
the cat *J Physiol* (Lond) 1968 *196* 19—35
arge muscle afferents from the cat s forelimb *Acta*
ors and afferent fibres of the forelimb interosseous nerve of the cat *Acta physiol scand* 1970 *79* 6—23
Skoglund S Anatomical and physiological studies of knee joint innervation in the cat *Acta physiol scand* 1956 *36* Suppl 124
Swett J E and C M Bourassa Short latency activation of pyramidal tract cells by Group I afferent volleys in the cat *J Physiol* (Lond) 1967 *189* 101—117
Uddenberg N Differential localization in dorsal funiculus of fibres originating from different receptors *Exp Brain Res* 1968 *4* 367—376

Acta physiol. scand. 1970. 80. 215—221

From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Quantitative Correlation between Degranulation and Histamine Release Following Exposure of Rat Mast Cells to Compound 48/80 in vitro

By

RADOMÍR NÓSAL, STUART A SLORACH and BÖRJE UVNÄS

Received 24 February 1970

Abstract

NOSÁL, R, S A SLORACH and B UVNÄS *Quantitative correlation between degranulation and histamine release following exposure of rat mast cells to compound 48/80 in vitro* Acta physiol scand 1970 *80* 215—221

The relationship between the release of histamine and ^{35}S labelled heparin from mast cells exposed to compound 48/80 *in vitro* has been studied in order to elucidate the mechanism of histamine release induced The cells were taken from rats injected 3 6 12 or 20 days earlier with $Na_2{}^{35}SO_4$ A good correlation was found between the two release curves From studies [illegible] 48/80 involves an initial extrusion of histamine cotnaining granules followed by an exchange of histamine in the extruded granules and cations in the extracellular medium

In rat mast cells the histamine is stored in discrete intracellular granules which mainly consist of a protein-heparin complex The exposure of such cells to compound 48/80 results in the extrusion of granules and the release of histamine It has previously been proposed from this laboratory (Uvnäs 1964, Uvnas and Thon 1966 Thon and Uvnas 1967), that the response of mast cells to compound 48/80 is essentially a two-stage process The initial event, the degranulation, is an energy-requiring extrusion of histamine-containing granules The second stage is an extracellular exchange between histamine in the extruded granules and cations in the extracellular medium

Using mast cells isolated from rats injected two days earlier with $Na_2{}^{35}SO_4$, Fillion, Slorach and Uvnas (1970) showed that there was a correlation between the release of histamine, ^{35}S-heparin and granule protein following exposure to compound 48/80 *in vitro* this is consistent with the above hypoth [illegible]

the percentage histamine released exceeded the percentage of ^{35}S-labelled granules extruded, the possibility of some additional release process could not be excluded. Since the discrepancy between the percentages of histamine released and ^{35}S-labelled granules extruded might have been due to an uneven labelling of the granules with ^{35}S due to the short labelling time we have extended these studies. We allowed different intervals of time between the $Na_2{}^{35}SO_4$ injection and isolation of the cells and studied in detail the relationship between ^{35}S and protein in the extruded granules and granules remaining in the cells after exposure to compound 48/80. In addition, we studied the time courses of histamine and ^{35}S-labelled granule release after exposure of labelled mast cells to compound 48/80.

Methods and materials

Labelling and isolation of mast cells

Mast cells were labelled and isolated as described by Fillion *et al.* (1970) except that the rats were injected with 2 mCi $Na_2{}^{35}SO_4$ s.c. at different times (3, 6, 12 or 20 days) before taking the mast cells. Examination of the localization of radioactivity in the different cell constituents of mast cells taken from rats injected 29 days earlier, using methods described by the above authors, showed that the ^{35}S was localized almost exclusively in heparin molecules. Since this was also the case two days after injection it is assumed that the radioactivity in the mast cells used in these studies is virtually all present in heparin molecules.

Attempts to improve the recovery of extruded granules

^{35}S-labelled mast cells (660,000 and 615,000/ml) from rats injected 6 days earlier were incubated for 5 min in the presence or absence of compound 48/80 (1 μg/ml). The effect of adding glucose (1 mg/ml, preincubation 10 min, 20° C) and/or using 0.34 M sucrose adjusted to pH 7.8 for the last two 2 ml washes of the cells was studied. Apart from these changes all procedures were carried out as described below.

From Fig. 1 it can be seen that the addition of glucose to the incubation medium did not affect either the histamine or the ^{35}S release from untreated or compound 48/80-treated mast cells. However, using 0.34 M sucrose for the last two washes to remove adhering granules resulted in a better recovery of the ^{35}S-labelled granules and consequently a reduction in the ratio percentage histamine release/percentage ^{35}S release. There was also a very small increase in the histamine release in both the untreated and the treated cells washed with sucrose. Since we wished to minimize the spontaneous release of histamine it was considered inadvisable to use sucrose and the washings in the experiments below were carried out using salt solutions as described later.

Effect of different labelling times on the ratio histamine release/^{35}S release

^{35}S-labelled mast cells (7–8 × 10^5/ml) taken from rats injected 6, 12 or 20 days earlier were incubated (10 min, 37° C) with compound 48/80 (0–10 μg/ml) and washed (3 × 2 ml salt solution pH 7.8 containing human serum albumin) as described by Fillion *et al.* (1970) — "general method". The release of histamine, ^{35}S and granule protein was determined as described by the above authors except that the granules were washed with three 2 ml aq. dest. washes instead of two.

^{35}S/protein ratio in granules

The ratio ^{35}S (cpm) to protein (μg pepsin standard) was determined in the isolated extruded granules. Protein was determined by the method of Lowry *et al.* (1951) and is expressed in terms of the crystalline pepsin standard used. In addition, the water-lysed cells (previously exposed to compound 48/80 and washed to remove adhering granules) were adjusted to pH 7.8, centrifuged (350 × g, 10 min, 4° C) and the supernatant was recentrifuged (2700 × g, 30 min, 4° C). The resulting precipitate (granules) was washed three times with 2 ml quantities of distilled water. The ratio ^{35}S/protein was then determined after dissolving the granules in 2 ml of 0.05 N NaOH.

From the above experimental data it was also possible to calculate the release of granule protein per μg of histamine released.

Fig 1 The effect of adding glucose and/or washing with 0 34 M sucrose on the release of histamine (whole bars) and ^{35}S (hatched part of bars) from mast cells with or without compound 48/80 (1 μg/ml) Each value is the mean of 2 expts duplicate samples were taken in each experiment Vertical bars indicate range

Time course of histamine and ^{35}S release following exposure to compound 48/80

^{35}S-labelled mast cells (687 500) obtained from rats injected three days earlier were pre-

Preparations used

Compound 48/80 was kindly supplied by Dr B Hogberg AB Leo Halsingborg Sweden Sulphur 35 as carrier free sulphate in sterilized neutral isotonic solution was obtained from the Institutt for Atomenergi, Kjeller Norway and recrystallized pepsin from E Merck AG, Darmstadt, W Germany All other materials were obtained from normal commercial sources

Results

Effect of labelling time on

a) Ratio percentage histamine release/percentage ^{35}S release following exposure to compound 48/80

Fig 2 shows the ratio percentage histamine release/percentage ^{35}S release from mast cells taken from rats injected 6, 12 or 20 days earlier with $Na_2{}^{35}SO_4$ The release in cells not exposed to compound 48/80 has been deducted before calculating the ratio

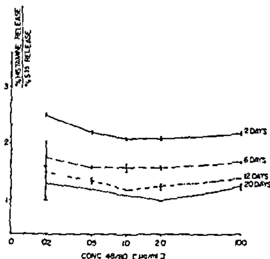


Fig 2

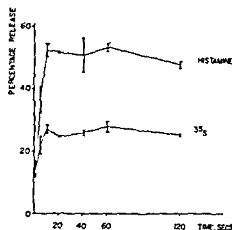


Fig 3

Fig 2 Effect of different labelling times on the ratio histamine release/^{35}S release after exposure to compound 48/80 Except for the 2 day curve each point is the mean of duplicates vertical bars indicate range 2 day curve from Fillion *et al* (1970) (*vide* Results) 5 expts duplicates in each vertical bars indicate S D

Fig 3 Time courses of histamine and ^{35}S release from labelled mast cells exposed to compound 48/80 (1 µg/ml) at 37° C labelling time 3 days Each point is the mean of duplicates vertical bars indicate range

The curve for the two day interval was calculated from data from Fillion *et al* (1970) and unpublished data from the same authors It can be seen that there is a progressive decrease in the ratio with increasing labelling time approaching unity after 12 or 20 days

b) Ratio ^{35}S/protein in extruded granules and granules remaining in the cells after compound 48/80 treatment

From Table I it can be seen that with an interval of 6 days between injection and taking the mast cells the ratio ^{35}S (cpm)/protein (µg pepsin standard) in the extruded granules is lower than that in granules obtained by water lysis of the washed compound 48/80 treated cells When the labelling time is increased the distribution of ^{35}S with respect to the granule protein becomes more even so that after 12 or 20 days the ratio ^{35}S/protein in the extruded and non extruded granules is very similar The disrupted cell suspension was adjusted to pH 7 8 before isolating the granules so that both they and the extruded granules had been exposed to the same pH The differences in the absolute values of the ratios from one experiment to another is of no significance since different batches of cells were used in each experiment

TABLE I Ratio ³⁵S (cpm) / Protein (μg pepsin) in granules

Granule source	Time	Interval	(Days)
	6	12	20
a) Extruded after exposure to compound 48 80			
0 2 μg/ml	145 (141—149)	195 (186—204)	116 (114—118)
0 5 μg/ml	168 (168—169)	215 (214—216)	128 (126—130)
1 0 μg/ml	181 (180—182)	222 (210—234)	131 (126—137)
2 0 μg/ml	166 (161—170)	225 (221—230)	134 (133—135)
10 0 μg/ml	189 (188—191)	216 (210—222)	127 (127—128)
b) Obtained from water lysed cells adjusted to pH 7 8	249* (244—253)	214 (208—220)	127 (121—133)

Each figure is the mean of duplicates or triplicates*, figures in brackets indicate range

Release of granule protein

The release of granule protein expressed as μg of the pepsin standard per μg histamine released is shown in Table II Within each experiment the release of granule protein per μg of histamine released is fairly similar at all concentrations of the releaser This is in agreement with the findings of Fillion *et al* (1970) the absolute quantity of protein released per μg histamine is also in the same range as previously found

TABLE II Release of protein (μg pepsin) per μg histamine released

Conc 48/80 (μg/ml)	Time	Interval	(Days)
	6	12	20
0	4 1 (4 1—4 0)	3 0 (2 7—3 4)	2 7 (2 2—3 2)
0 2	4 2 (4 2—4 3)	2 9 (2 6—3 1	2 8 (2 7—2 8)
0 5	4 0 (4 0—4 1)	3 0 (3 0—3 0	3 1 (3 0—3 2)
1 0	3 6 (3 5—3 7)	3 1 (3 0—3 1)	3 6 (3 3—3 9)
2 0	4 3 (4 3—4 3)	3 1 (3 1—3 1)	3 9 (3 9—4 0)
10 0	3 4 (3 4—3 4)	3 1 (3 1—3 2)	3 8 (3 7—3 9)
Mean	3 9	3 0	3 3

Each figure is the mean of duplicates figures in brackets indicate range

Time courses of histamine and ^{35}S release

Fig 3 shows the time courses of the release of histamine and ^{35}S following exposure of ^{35}S labelled mast cells to compound 48/80 (1 µg/ml) at 37° C Untreated cells incubated for 120 sec released 13 8 % of their histamine (range 13 3—14 3) and 11 6 % of their ^{35}S (range 10 2—13 1) There is a good correlation between the two release curves which is consistent with the hypothesis that the two processes de granulation and histamine release are intimately connected The cells were taken from rats injected 3 days earlier with $^{35}SO_4$ The mean ratio per cent histamine release/per cent ^{35}S release was 1 8

Discussion

In a previous paper from this laboratory (Fillion *et al* 1970) evidence of a quantitative correlation between the release of histamine ^{35}S labelled heparin and granule protein from labelled mast cells exposed to compound 48/80 *in vitro* was presented It was concluded that the principal mechanism of histamine release induced by compound 48/80 involves degranulation as proposed by Thon and Uvnas (1967) However since the percentage of histamine released exceeded the percentage of ^{35}S released the possibility of an additional release mechanism could not be excluded In those studies the cells were taken from rats injected two days earlier with 4 mCi $Na_2{}^{35}SO_4$ Virtually all the isotope was present in heparin molecules after this time Comparison of the ratio ^{35}S/protein in extruded granules and granules remaining in the cells after compound 48/80 treatment showed no apparent differences Therefore it was assumed that the ^{35}S was evenly distributed among the granules and that the release of ^{35}S would give a good guide to the release of granules However recent studies (Nosal Slorach and Uvnas to be published) have shown that there is a loss of protein from isolated granules when exposed to increasing pH Thus there is a progressive increase in the ratio ^{35}S/protein with increasing pH When the comparison was made in the previous paper the granules obtained by water lysis of the washed compound 48/80 treated cells were not exposed to the same pH as the extruded granules washed from the cells using a solution buffered to pH 7 8 (a suspension of mast cells lysed in distilled water usually has a pH of 6—6 5) If the ratios had been compared at the same pH a difference would probably have been evident In the present experiments where all the granules were exposed to the same pH (7 8) it was found that even after a labelling time of 6 days the distribution of ^{35}S was not uniform there being a preferential labelling of the non extruded granules However, after 12 or 20 days the labelling appeared essentially uniform and no difference was seen in the ratio ^{35}S/protein in extruded granules and those obtained by water lysis of compound 48/80 treated cells at pH 7 8

A progressive reduction in the ratio percentage histamine release/percentage ^{35}S release was found with increasing time between injection and cell isolation At times when the ^{35}S labelling may be considered as even and it is justifiable to use ^{35}S

release as a guide to granule release (12 or 20 days) the ratio lies in the range 1 1—1 4

In presenting the present results we have made a correction for the cells damaged during the washing procedures by deducting the histamine and ^{35}S release occurring in untreated cells from all values before calculating the ratio As discussed previously by Fillion *et al* (1970), this ratio would be further reduced if it were possible to isolate all the extruded granules

We conclude from these studies that there is a good quantitative correlation between the proportion of granules and histamine released on exposure of mast cells to compound 48/80 *in vitro* The time courses of the release of granules and histamine were also found to be similar If histamine in mast cells is evenly distributed among the granules then our results support the hypothesis that histamine release induced by compound 48/80 involves an initial extrusion of histamine containing granules followed by an extracellular cation exchange This appears to be true over the whole concentration range studied and we have no evidence for any histamine release taking place without a corresponding extrusion of granules The fact that the ratio histamine release/^{35}S release is slightly higher at the lowest concentration of releaser is probably due to the technical difficulties in collecting a high percentage of the relatively few granules extruded

This investigation was supported by a grant from the Swedish Medical Research Council (B69 14X 39 05A)

References

FILLION G M B S A SLORACH and B UVNÄS The release of histamine heparin and granule protein from rat mast cells treated with compound 48/80 *in vitro* *Acta physiol scand* 1970 *78* 547—560

LOWRY O H N J ROSEBROUGH A L FARR and R J RANDALL Protein measurement with the Folin phenol reagent *J biol Chem* 1951 *193* 265—275

THON I L and B UVNÄS Degranulation and histamine release two consecutive steps in the response of rat mast cells to compound 48/80 *Acta physiol scand* 1967 *71* 303—315

UVNÄS B Release processes in mast cells and their activation by injury *Ann N Y Acad Sci* 1964 *116* 880—890

UVNÄS B and I L THON A physico-chemical model of histamine release from mast cells Proc int Wenner Gren Center Sympos Stockholm 1965 *Mechanisms of Release of Biogenic Amines* Ed von Euler Rosell and Uvnäs Pergamon Press Oxford 1966 *pp* 267—289

Acta physiol scand. 1970 80 222—234

From the Department of Pharmacology, Kungl Veterinärhögskolan Stockholm Sweden

Effects of Increased Liver Metabolism of Nicotine on Its Uptake, Elimination and Toxicity in Mice

By

TORBJÖRN STÅLHANDSKE

Received 24 February 1970

Abstract

STÅLHANDSKE T *Effects of increased liver metabolism of nicotine on its uptake, elimination and toxicity in mice* Acta physiol scand 1970 80 222—234

Male albino mice were injected intraperitoneally or intravenously with ^{14}C-labelled nicotine Both untreated and phenobarbital pretreated mice were used The concentrations of nicotine and metabolically formed cotinine were determined in the brain liver and blood 1 2 5, 5 10 20 and 60 min after injection It was found that phenobarbital pretreated mice exhibited an enhanced liver metabolism of nicotine both in vitro and in vivo The increased liver metabolism caused a significant decrease of nicotine concentration in the brain only when nicotine was given intraperitoneally Phenobarbital pretreatment elevated the intraperitoneal LD_{50} value 2—3 times and also increased tolerance to repeated sublethal doses of nicotine No change in the intravenous LD_{50} value was observed

The dose of nicotine necessary for producing certain toxic effects in vivo varies considerably with the way of administration This difference is not only due to different absorption conditions but also to different routes of transportation to target organs

This fact was for the first time illustrated by Lautenbach (1876—1877) who demonstrated that a nicotine dose lethal to dogs when given into general circulation, was sublethal when given into mesenteric and splenic veins and thus had to pass the liver before reaching the general circulation Later findings by Hansson and Schmiterlow (1962) showed that nicotine is easily taken up by the liver which is the most active organ for nicotine detoxication (for review see Larson *et al* 1961) Consequently when nicotine has to pass the liver before entering the general circulation this organ obviously influences the amount of nicotine transported to different target organs

Many of the acute toxic effects of nicotine is known to emanate from its action in the central nervous system (for review see Silvette *et al* 1962) A high affinity for nicotine in brain tissue with a specific localization to nerve cells was demonstrated by autoradiographical means by Schmiterlow *et al* (1967) They also showed that the molecular and pyramidal cell layers of hippocampus had a particularly high concentration of nicotine

The aim with the present investigation was to evaluate the influence of the liver on nicotine toxicity and nicotine uptake in the brain after intravenous and intraperitoneal administrations. Drugs injected into the peritoneal cavity are primarily absorbed through portal circulation and thus have to pass the liver before reaching general circulation (Lukas 1967).

Present findings demonstrated a significantly increased ability of the liver to metabolize nicotine after phenobarbital pretreatment. Hence it was also possible to evaluate the influence of different rates of nicotine metabolism in the liver on the uptake and toxicity of nicotine.

Materials and methods

Compounds

Nicotine methyl ^{14}C was synthesized according to the method by McKennis *et al.* (1962) as described by Hansson and Schmiterlöw (1962). The specific activity of different syntheses varied between 28 and 46 μCi/mg.

Experimental animals

Male albino mice weighing 18—22 g were used. Pretreated animals had been treated with intraperitoneal injections of phenobarbital sodium 100 mg/kg dissolved in distilled water once daily for 3 days. Animals were starved and used 18—20 hrs after the last injection. Control animals had been injected with the same volume of distilled water 0.1 ml/20 g b.w.

In vivo studies

The animals were injected intravenously or intraperitoneally with ^{14}C-nicotine dissolved in distilled water. The injected nicotine dose was 1.0 mg/kg and the volume 0.1 ml/20 g body

and blood were removed and weighed. The tissues were immediately frozen and kept at −20° C until determination of nicotine and cotinine. The number of animals at each time varied between 3 and 9.

In one experiment the hippocampal formation was separated from the rest of the brain in 6 animals 10 min after intravenous injection of nicotine and analyzed for nicotine separately.

Urinary collection

nicotine injections. The average urinary pH was about 5.5.

Extractions

Brains and livers were homogenized in 0.2 M phosphate buffer pH 7.4 and the homogenates, blood and urine were extracted with heptane and chloroform (Hucker *et al.* 1960) and thus

the use of thin layer chromatography (Stålhandske 1970). All determinations of radioactivity were carried out for sufficient time to yield at least 1000 counts. Values in cpm were converted to values in μg per sample based on the specific activity of the ^{14}C-nicotine used and on absolute counting efficiencies determined with standards of ^{14}C-toluene.

In vitro studies

[illegible]

supernatants, the content of the incubation mixtures and the methods used for the determination of reaction rates have been described previously (Stålhandske 1970) Nicotine was added to the incubation mixture in a concentration of 1 6 mM and under the assay conditions used the enzymatic reaction was linear with time and protein content

The protein concentration of the supernatants was determined by the Biuret method (Szarkowska and Klingenberg 1963) Crystalline serum albumine was used as a standard

Statistical evaluation of data

Student's "t" distribution was used as a test of the null hypothesis The level of significance used was P less than 0 05

Acute toxicity

Animals in a control and phenobarbital pretreated group were injected i p and i v with nicotine bitartrate in distilled water I v doses of 0 2, 0 4, 0 5, 0 6 0 8 and 1 mg/kg and i p doses of 5 10 15 20, 30 40 50 and 60 mg/kg b w calculated as nicotine base were used

In all groups 10 animals were injected with each dose of nicotine and the injected volume was 0 1 ml/20 g b w *The calculations were based on the number of animals dead 1/2 hr after* injection After this time further deaths very rarely occurred The LD_{50} and its confidence limits for 95 per cent probability were determined by the method of Litchfield and Wilcoxon (1949)

In another experiment a control and a phenobarbital pretreated group each representing 40 mice received i p 5 15 mg/kg body weight calculated as nicotine base every fifth minute The number of animals dead immediately prior to the next time scheduled injection was observed The number of doses required to cause death has been used as a measure of the effects of the phenobarbital pretreatment on nicotine toxicity This method was suggested by Larson *et al* (1945)

Results

Distribution of nicotine

Intravenously injected nicotine (Fig 1) was very rapidly taken up in the brain and a maximum concentration was observed as early as 1 min after injection. At that time a maximum concentration of nicotine was also observed in the blood Later on the concentrations of nicotine decreased rapidly In the brain it was found to be less than 50 % of its maximum as early as 5 min after injection and at 60 min it had decreased to only 1 %

The nicotine concentration in the hippocampal formation 10 min after i v injection was on an average 32 1 % higher than in the rest of the brain The difference was significant (Table I)

One min after injection the ratio between nicotine concentrations in brain and blood was 5 6 This ratio remained relatively constant in later observations but at 60 min it was decreased (Table II) In all observations the nicotine concentration in the brain was considerably higher than in the liver The nicotine concentration reached a maximum in the liver 5 min after injection At that time the liver to blood ratio was 2 3

Intraperitoneally injected nicotine was very rapidly taken up in liver A maximum concentration was seen as soon as 2 5 min after injection (Fig 2) At that time the blood concentration had also reached a maximum and the liver to blood ratio was as high as 14 5

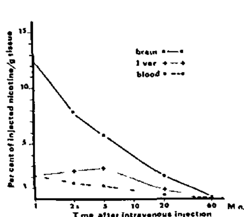


Fig 1

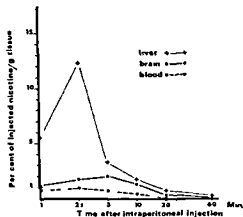


Fig 2

Fig 1 Nicotine concentrations in the brain liver and blood of mice various times after intra venous injection of ^{14}C nicotine Each value represents the average of at least 3 animals

Fig 2 Nicotine concentrations in the brain liver and blood of mice various times after intra peritoneal injection of ^{14}C nicotine Each value represents the average of at least 3 animals

TABLE I Per cent of injected nicotine g in the hippocampal formation and the rest of the brain 10 min after i v injection

Animal No	Hippocampal formation %	Rest of the brain %	Difference
1	4 01	2 45	1 56
2	3 92	3 09	0 83
3	2 98	2 59	0 39
4	3 38	2 70	0 68
5	5 81	4 17	1 64
6	5 56	4 56	1 00
			Mean 1 02 ± 0 20 (S E M) $p<0.005$

TABLE II Time course of brain to blood levels

Minutes after injection	Intravenous injection		Intraperitoneal injection	
	Controls	Pretreated*	Controls	Pretreated*
1	5 6	5 6	1 6	1 2
2 5	5 2	6 1	1 9	1 9
5	5 1	4 6	3 0	2 5
10	—	—	2 9	2 7
20	5 8	5 5	2 2	2 2
60	4 0	3 4	2 7	2 4

Each result represents the average of at least 3 animals

* Pretreatment see methods

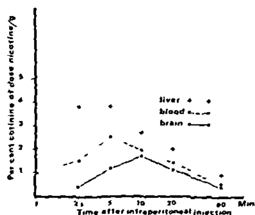


Fig 3

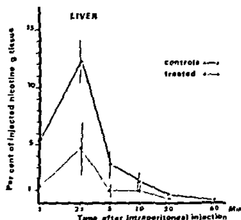


Fig 4

Fig 3 Cotinine concentrations in the liver, blood and brain of mice various times after intra peritoneal injection of ^{14}C-nicotine Each value represents the average of at least 3 animals

Fig 4 Nicotine concentrations in livers of controls and phenobarbital pretreated animals various times after intraperitoneal injection of ^{14}C-nicotine The values in the figure represent the average of at least 3 animals Vertical bars are standard deviations

Average nicotine concentrations ± S E M in livers

Min after inj	Number of exp	Controls	Number of exp	Pretreated	p
1	4	5 50 ± 0 44	4	1 47 ± 0 33	<0 001
2 5	5	12 26 ± 0 83	5	4 66 ± 1 06	<0 001
5	9	3 28 ± 0 42	9	1 01 ± 0 20	<0 001
10	9	1 76 ± 0 28	7	0 98 ± 0 19	<0 05

In contrast to the observations after i v injection the concentration in the liver was higher than in the brain in all observations In the brain nicotine concentrations reached a maximum 5 min after injection and the maximum brain to blood ratio (3 0) was then lower than after i v injection

Cotinine formation

After injecting nicotine *intraperitoneally* a maximum concentration of cotinine in the liver was observed as early as 2 5 min (Fig 3) This concentration remained at 5 min but later it decreased rapidly A maximum cotinine concentration in the blood after i p injection was not observed until 5 min and in the brain 10 min after injection Both after i v and i p injections the ratio between maximum average concentrations of cotinine in liver blood and brain was found to be 3 7 1 5 1 0 respectively However, maximum cotinine concentrations after i p injection were 3 times higher than after i v injection of nicotine

After intravenous injection a maximum cotinine concentration in liver was not seen until 5 min after injection and the ratio of cotinine concentrations in brain and blood was in all observations below 1

TABLE III

	Number of assays	Liver weight[a]	Cotinine formed µmoles/hr/100 mg protein[a]	Number of assays	$^{14}CO_2$ formed in pro mille of added radioact[b]
Controls (water injections)	12 (4)	0 98 ± 0 05	2 49 ± 0 09	3 (1)	0 18 ± 0 06
		p < 0 05	p < 0 001		
Pretreated*	12 (4)	1 17 ± 0 04	6 29 ± 0 20	4 (1)	0 42 ± 0 07

Effects of phenobarbital treatment on the liver metabolism of nicotine to cotinine and CO_2 in vitro Results are a) mean ± S E M b) mean ± S D
Incubations were made from a pool of 3 livers Number of liver pools are shown in brackets

* Pretreatment see methods

Effect of phenobarbital pretreatment

Liver metabolism of nicotine in vitro

I p injections of 100 mg/kg of phenobarbital once a day for 3 days enhanced the in vitro liver metabolism of nicotine There was a significantly increased formation of the major metabolite cotinine and also an increased formation of CO_2 (Table III)

In vivo

The phenobarbital pretreatment increased the liver weight significantly (Table III) A maximum concentration of nicotine in the livers of phenobarbital pretreated animals was as in controls seen 2 5 min after i p injection (Fig 4) The same figure shows that the average nicotine concentration in livers of pretreated animals in all observations was lower than in livers of controls The nicotine concentrations were significantly lower in observations 1, 2 5 5 ($p < 0 001$) and 10 min ($p < 0 05$) after injection During the period of observation the nicotine concentrations in livers of pretreated animals were on an average 63 8 % lower than in livers of controls

A similar decrease in liver concentrations of nicotine in pretreated animals was also seen after i v injection In this case the nicotine concentrations in pretreated animals were on an average 44 4 % lower than in controls (Fig 5)

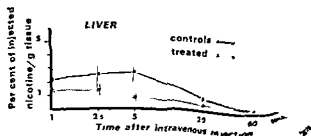


Fig 5 Nicotine concentrations in livers of controls and phenobarbital treated animals various times after intravenous injection of ^{14}C nicotine Each value represents the average of at least 3 animals Vertical bars are standard deviations

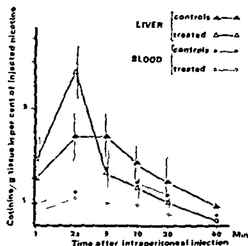


Fig 6

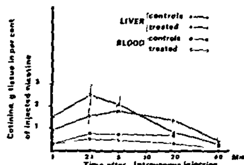


Fig 7

Fig 6 Cotinine concentrations in liver and blood of controls and phenobarbital pretreated animals various times after intraperitoneal injection of ^{14}C-nicotine Each value in the figure represents the average of at least 3 animals Vertical bars are standard deviations

Average cotinine concentrations ± S E M in livers

Min after inj	Number of exp	Controls	Number of exp	Treated	p
2 5	5	3 85 ± 0 46	5	6 57 ± 0 50	<0 01
5	9	3 86 ± 0 33	8	2 32 ± 0 37	<0 02

Fig 7 Cotinine concentrations in liver and blood of controls and phenobarbital pretreated animals various times after intravenous injection of ^{14}C-nicotine Each value represents the average of at least 3 animals Vertical bars are standard deviations

Higher *cotinine* concentrations were found in livers of phenobarbital pretreated animals than in controls 1 and 2 5 min after injection (Fig 6)

At 2 5 min the cotinine concentrations in livers of pretreated animals were on an average 70 % higher ($p<0\,01$) than in controls However at 5 min after injection the cotinine concentrations in livers of pretreated animals had rapidly decreased and were on an average 40 % lower ($p<0\,02$) than in controls Also in later observations the cotinine concentration in livers of pretreated was lower than that in control animals

As is shown in Fig 7 a similar relation between cotinine concentrations was also seen after i v injection

The cotinine concentration in blood was in all observations lower in pretreated animals (Fig 6 and 7

Nicotine distribution in brain and blood

As is seen in Fig 8 the average nicotine concentration in blood was lower in phenobarbital pretreated animals than in controls in all observations both after i v and i p

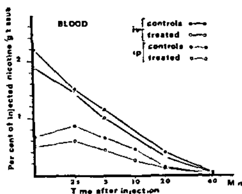


Fig 8

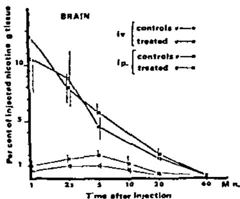


Fig 9

Fig 8 Nicotine concentrations in blood of control and phenobarbital pretreated animals various times after intraperitoneal and intravenous injections of ^{14}C-nicotine The values represent the average from 3 animals

Fig 9 Nicotine concentrations in brains of controls and phenobarbital pretreated animals various times after intravenous and intraperitoneal injections of ^{14}C-nicotine The values represent the average of at least 3 animals Vertical bars are standard deviations

Average nicotine concentrations ± S E M in brains

	Min after inj	Number of exp	Controls	Number of exp	Pretreated	p
Intravenous	2 5	5	7 94 ± 0 59	4	8 82 ± 1.26	
injection	5	5	5 84 ± 0 31	4	4 54 ± 0 57	> 0 1
	2 5	5	1 65 ± 0 17	5	1 03 ± 0 06	< 0 05
Intraperitoneal	5	8	2 01 ± 0 16	8	1 08 ± 0 07	< 0 001
injection	10	8	1 32 ± 0 26	7	0 59 ± 0 17	< 0 05

injections of nicotine The pretreated animals had on an average 10 and 29 % lower blood concentrations than in controls after i v and i p injections respectively

In the brain nicotine concentrations were in all observations lower in pretreated animals after i p injection On an average the concentrations in the brain of pretreated animals were 37 7 % lower than in controls In separate observations a significant difference was observed at 2 5 10 (p < 0 05) and 5 (p < 0 001) min after injection (Fig 9)

After i v injection no significant differences of nicotine concentrations in brain were observed (Fig 8)

Urinary excretion

Lower amounts of unchanged nicotine and formed cotinine were excreted in urine of phenobarbital pretreated animals after i p injection of nicotine However, a higher excretion of water soluble metabolites was observed in pretreated animals (Fig 10)

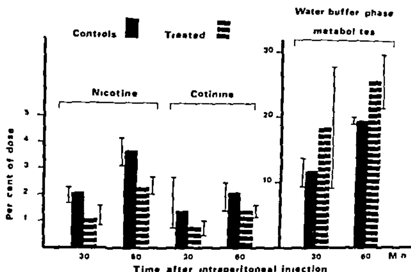


Fig 10 The urinary excretion of nicotine cotinine and water buffer phase metabolites 30 and 60 min after intraperitoneal injection of ^{14}C n cotine in controls and phenobarbital pretreated animals Each value represents an average of 3 expts and bars represent extremity values

Acute toxicity

The LD_{50} values of nicotine in controls and phenobarbital pretreated animals after i v and i p injections are shown in Table IV

A significant difference was observed after i p injection where LD_{50} for pretreated animals is 2 4 times higher than in controls No significant difference was observed after i v injection

Twelve i p injections of a sublethal dose of nicotine (5 15 mg/kg) given every fifth minute killed 100 % of the control animals However the same number of injections only killed 22 % of the phenobarbital pretreated animals To kill 100 % of the pretreated animals 28 injections were needed (Fig 11)

TABLE IV Acute toxicity

Administration	LD_{50} (mg/kg body weight)			
	Number of mice	Controls	Number of mice	Pretreated*
Intravenous	60	0 60 (0 71—0 51)	60	0 50 (0 65—0 39)
Intraperitoneal	50	14 (11 6—15 8)	60	34 (27 8—41 5)

Figures in brackets are 95 % confidence limits

* Pretreatments see methods

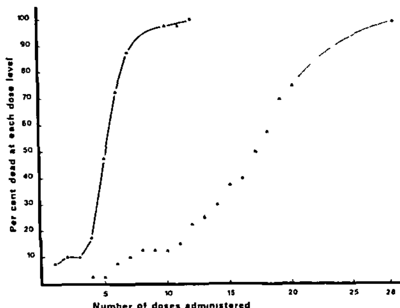


Fig 11 Influence of phenobarbital pretreatment on the number of sublethal doses of nicotine (5 15 mg/kg) tolerated by mice The dose was administered intraperitoneally every fifth minute Each group represents 40 mice ▲—▲ Controls ▲ ▲ Phenobarbital pretreated

Discussion

A maximum nicotine concentration in the brain was observed as early as 1 min after i v injection and ratios between brain and blood concentrations after i v injection reveal that equilibrium between brain and blood concentrations occurred within 1 min after the administration The brain/blood ratio was then as high as 5 6 These data show that nicotine passes into the brain with extraordinarily rapidity apparently unhindered by the blood brain barrier

In agreement with the autoradiographical findings by Schmiterlow *et al* (1967) the present experiments showed a significantly higher concentration of nicotine in the hippocampal formation These findings may serve as an explanation to results by Dunlop *et al* (1960) and Floris *et al* (1964) who demonstrated that hippocampus in comparison with cortical and reticular formations has the lowest threshold for nicotine effects

I p administered nicotine is very rapidly absorbed since maximum concentrations in tissues were seen within 5 min after the injection The nicotine concentration in the liver was considerable as early as 1 min after the injection This concentration was higher than ever observed after i v injection

Hansson *et al* (1964) who investigated the in vitro metabolism of nicotine in various organs of mice found no metabolism of nicotine in brain tissue slices They found that the liver was the principal organ concerning nicotine metabolism Schmi

terlow *et al* (1967) suggested that the small amounts of cotinine found in the brain are formed in the liver and transported to the brain

This suggestion is further supported by the present findings showing that maximum cotinine concentration was first observed in the liver, later in the blood and last in the brain The blood concentrations were always lower than liver concentrations and higher than brain concentrations The fact that the maximum concentration of cotinine in blood was lower and delayed in comparison with the maximum liver con centration indicates that there exists a hindrance for cotinine transport to blood and that parts of formed cotinine probably are bound for further metabolism (Morselli *et al* 1967 Stålhandske 1970)

The higher and earlier cotinine maximum in blood in comparison with brain indi cates that cotinine transfer through blood brain barrier is slower and brain uptake is lower than for nicotine (Bowman *et al* 1964) The higher cotinine concentrations seen in liver and other organs after i p injection are probably due to the better satura tion of enzymes in liver with nicotine

The observation of considerable amounts of cotinine as early as 1 min after i p in jection showed that the metabolism of nicotine to cotinine is extremely rapid in vivo

Phenobarbital known as a well documented inducer of many microsomal enzymes (for review see Mannering 1968) was also found to stimulate the metabolism of nicotine In agreement with findings by Kunz *et al* (1966) the phenobarbital pre treatment also increased the liver weight In vitro an increased hydroxylation of nicotine to cotinine and an increased formation of ^{14}CO was observed Referring to the label of ^{14}C nicotine used the increase of ^{14}CO released indicates that pheno-barbital induces the N demethylation of nicotine (McKennis *et al* 1962 Stålhandske 1970)

*In vivo significantly lower concentrations of nicotine were seen in livers of pheno-*barbital pretreated animals In early in vivo observations 1 and 2 5 min after injec tion as in in vitro experiments higher amounts of cotinine were formed in livers o pretreated animals These observations indicate that the lower nicotine levels are du to a more rapid liver metabolism of nicotine in pretreated animals Thus the pheno barbital pretreated animal has an increased ability to decrease the amount of nicotin passing through the liver

In observations 5 min after injection and later lower cotinine concentrations were seen in livers of pretreated animals Consequently cotinine is not only formed faster but also disappears faster in pretreated animals This may be due to several factors acting together in a dynamic sequence

Cotinine metabolism was also stimulated by the pretreatment Due to higher coti nine concentrations cotinine metabolizing enzymes were for a short time better saturated and worked more effectively in pretreated animals Due to the more rap d disappearance of nicotine in pretreated animals the saturation of enzymes decreased faster to unfavourable levels and hence the cotinine formation initially high rapidly ceased

The suggestion above that phenobarbital pretreatment both stimulates nicot ne

and cotinine breakdown in vivo was further supported in the excretory studies The urine of pretreated animals was found to contain lower concentrations of both nicotine and cotinine but higher concentrations of other metabolites some shown to be formed by the metabolism of cotinine *in vivo* (Bowman *et al* 1963)

Those findings may explain results by Beckett and Triggs (1967) who studied excretion of nicotine in smokers and non smokers After administration of nicotine to smokers and non smokers they found that in smokers' urine less amounts of unchanged nicotine were seen than in that of non smokers They concluded that smokers had required a more rapid metabolism of nicotine However, they could not find any increase of cotinine excretion and therefore suggested that the increase of nicotine metabolism was not due to an increased formation of cotinine But in view of the present findings smokers may also have an increased metabolism of cotinine

The increased metabolism of nicotine observed in liver after phenobarbital pretreatment did only decrease brain concentrations of nicotine significantly after i p injection The absence of a decrease of nicotine concentrations in brains of phenobarbital pretreated animals after i v injection, is very likely due to the rapid passage of nicotine into brain and the high affinity of nicotine for brain tissue When nicotine is administered directly into general circulation as in i v injections the liver only gets parts of the injected nicotine in each round of circulation Nicotine which preferably accumulates in the brain is thus in the distribution phase only to a limited extent influenced by the increased liver metabolism

A decreased uptake of nicotine in brains of pretreated animals may be the cause of their augmented tolerance to intraperitoneally injected nicotine observed However, opinions are divided as to whether nicotine death is primarily provoked by a central or a peripheral action Nevertheless Aceto *et al* (1969) showed that some ganglion blocking agents exclusively present in the central nervous system are protective against the lethal effects of nicotine in mice and summarized that indirect evidences suggest that the sites of action of nicotine extensor convulsions and lethality is central in origin'

A protective effect against i p injected nicotine after pretreatment with chlorbutanol also having enzyme inducing properties (Kato and Chiesara 1962) was reported by Taber and Larson (1964)

This work was supported by grants to Professor Carl G Schmiterlow from the Swedish Tobacco Company the American Medical Association Education and Research Foundation and Knut och Alice Wallenbergs Stiftelse (for general equipment for isotope research work)

The skilful technical assistance of Mrs A Österblom and Mrs B Hagtorn is gratefully acknowledged

References

ACETO M D H C BENTELY and J R DEMBINSKI Effects of ganglion blocking agents on nicotine extensor convulsions and lethality in mice *Brit J Pharmacol* 1969 *37* 104—111

BECKETT A H and E J TRIGGS Enzyme induction in man caused by smoking *Nature* (Lond) 1967 *216* 587

BOWMAN E R E HANSSON L B TURNBULL H MCKENNIS JR and C G SCHMITERLÖW Disposition and fate of (—)-cotinine ³H in the mouse *J Pharmacol exp Ther* 1964 *143* 301—308

DUNLOP C W C STUMPF, D S MAXWELL and W SCHINDLER Modification of cortical reticular and hippocampal unit activity by nicotine in the rabbit *Amer J Physiol* 1960 *198* 515—518

FLORIS V, C MORCUTTI and G F AYALA Effects of nicotine on the cortical thalamic and hippocampal electrical activity in rabbits *J Neuropsychiat* 1964 *5* 247—251

HANSSON E and C G SCHMITERLOW Physiological disposition and fate of ^{14}C labelled nicotine in mice and rats *J Pharmacol exp Ther* 1962 *137* 91—102

HANSSON E P C HOFFMANN and C G SCHMITERLOW, Metabolism of nicotine in mouse tissue slices *Acta physiol scand* 1964 *61* 380—392

HUCKER H B J R GILLETTE and B B BRODIE Enzymatic pathway for the formation of cotinine a major metabolite of nicotine in rabbit liver *J Pharmacol exp Ther* 1960 *129* 94—100

KATO R and E CHIESARA Increase of pentobarbital metabolism induced in rats pretreated with some centrally acting compounds *Brit J Pharmacol* 1962 *18* 29—38

KUNZ W G SCHAUDE W SCHMID and M SIESS Stimulation of liver growth by drugs I Morphological analysis *Proc Europ Soc Study of Drug Toxicity* 1966 *VII* 113—137

LARSON P S H B HAAG and J K FINNEGAN On the relative toxicity of nicotine and nor nicotine *Proc Soc exp Biol* (N Y) 1945 *58* 231—232

LARSON P S H B HAAG and H SILVETTE *Tobacco Experimental and Clinical Studies* The Williams & Wilkins Company Baltimore 1961

LAUTENBACH B F On a new function of the liver *Philad Med Times* 1876—1877 *7* 387—394

LITCHFIELD JR J T and F WILCOXON A simplified method of evaluating dose effects experiments *J Pharmacol* 1949 *96* 99—113

LUKAS G S BRINDLE and P GREENGARD Route of absorption of intraperitoneally administered compounds *Fed Proc* 1967 *26* 615

MANNERING G J Significance of stimulation and inhibition of drug metabolism in pharmacological testing In *Selected Pharmacological Testing Methods* (Ed A Burger) Marcel Dekker Inc New York 1968 *3* 51—119

MCKENNIS JR H L B TURNBULL S L SCHWARTZ E TAMAKI and E R BOWMAN Demethylation in the metabolism of (—) nicotine *J biol Chem* 1962 *237* 541—546

MORSELLI P L H H ONG E R BOWMAN and H MCKENNIS JR Metabolism of (±) cotinine 2 ^{14}C in the rat *J med Chem* 1967 *10* 1033—1036

SCHMITERLOW C G E HANSSON G ANDERSSON L E APPELGREN and P C HOFFMANN Distribution of nicotine in the central nervous system *Ann N Y Acad Sci* 1967 *142* 2—14

SILVETTE H E C HOFF P S LARSON and H B HAAG The actions of nicotine on central nervous system functions *Pharmacol Rev* 1962 *14* 137—173

STÅLHANDSKE T The metabolism of nicotine and cotinine by a mouse liver preparation *Acta physiol scand* 1970 *78* 236—248

SZARKOWSKA L and M KLINGENBERG On the role of ubiquinone in mitochondria *Biochem Z* 1963 *338* 674—697

TABER R J and P S LARSON In vivo and in vitro studies on the question of a biologic relationship between nicotine and ascorbic acid *Arch int Pharmacodyn* 1964 *151* 243—259

Acta physiol. scand. 1970. 80. 235—248

From the Brain Research Laboratory, E blocket, University Hospital, and from the Department of Anesthesia, University of Lund, Sweden

The Effect of Anesthetics upon Labile Phosphates and upon Extra- and Intracellular Lactate, Pyruvate and Bicarbonate Concentrations in the Rat Brain

By

LORENTZ NILSSON and BO K. SIESJÖ

Received 26 February 1970

Abstract

NILSSON, L. and B. K. SIESJÖ. *The effect of anesthetics upon labile phosphates and upon extra and intracellular lactate, pyruvate and bicarbonate concentrations in the rat brain.* Acta physiol. scand. 1970. *80*. 235—248.

[illegible]

to give arterial CO_2 tensions of 35—40 mm Hg. It was found that the type or the depth of anesthesia did not significantly influence the tissue concentrations of phosphocreatine, ATP, ADP or AMP. There were very small differences in the tissue lactate concentration in the nonbarbiturate groups and thus no indication that the lactate concentration varied with the depth of anesthesia. However, in all barbiturate groups there was a lowering of the lactate concentration and an increase in the intracellular pH. In contrast to the volatile anesthetics the barbiturates gave significant decreases also in the calculated cytoplasmatic NADH/NAD+ ratio. The experiments thus failed to indicate that anesthetics inhibit electron transport or energy transfer reactions in the brain, at least under the conditions studied. In addition, they suggest that barbiturates, including amobarbital, shift the balance between energy yielding and energy requiring reactions in favour of an oxidated state.

There is an impressive literature about the effects of various anesthetics upon the energy metabolism of the brain and other tissues, covering both *in vivo* and *in vitro* experiments. Some of the *in vitro* work, carried out on tissue slices and on isolated mitochondrial systems, has suggested that barbiturates, and notably oxybarbiturates like amobarbital, inhibit electron transport and energy transfer reactions in the respiratory chain (Ernster *et al.* 1963, Chance and Hollunger 1963, Chance 1965). These results are in keeping with those obtained on unstimulated tissue slices (Quastel 1962, McIlwain 1966) as well as with results of *in vivo* measurements of the NADH fluorescence during administration of amobarbital (Chance *et al.* 1962).

Taken together, these results suggest that barbiturates lead to changes qualitatively similar to those occurring during hypoxia in that they involve increases in the NADH/NAD ratio and in the lactate concentrations as well as decreases in the phosphocreatine concentrations (see McIlwain 1966) It is thus not surprising that it has sometimes been suggested that anesthetics cut down the metabolic rate by restricting energy yielding reactions and that consequently, the anesthetic state is an energy depleted one

Clinical experience of the protective effects of barbiturates against tissue hypoxia as well as a number of *in vivo* studies, have failed to corroborate the results of the *in vitro* experiments Thus it has been found that changes in labile substrates in the brain during anesthesia with barbiturates or volatile anesthetics are the opposite of those occurring during hypoxia These changes which are also seen in stimulated tissue slices *in vitro* (McIlwain 1966), typically consist of decreases in the tissue lactate and AMP concentrations and of increases in the phosphocreatine concentrations (Stone 1938 Richter and Dawson 1948 Lowry *et al* 1961 Mayman *et al* 1964 Gatfield *et al* 1966 Goldberg *et al* 1966) Thus the results of the *in vivo* studies suggest that anesthetics primarily depress energy utilization in the brain and that anesthesia represents a high energy state

There are two types of criticism which can be directed towards most if not all of the previous studies of substrate levels in the intact brain Thus there are often reasons to assume that the changes in the substrate levels have occurred not as a result of the anesthesia itself but as an effect of the anesthetics on autolytic changes occurring during the freezing of the tissue (see Granholm *et al* 1968 Thus if anesthesia only slows down phosphocreatine breakdown and lactate production occurring during the fixation of the tissue the results obtained do not reflect steady state substrate levels It is clear that such artefactual changes are minimized if very small animals are used (Lowry *et al* 1964 Goldberg *et al* 1966) but the remaining effect is hard to quantitate Secondly most of the studies quoted have been carried out without control of the body temperature or of the arterial O_2 and CO_2 tensions The most critical parameter in this context may be the arterial CO_2 tension since even a moderate hyperventilation leads to an increase in the tissue lactate concentration (Leusen and Demeester 1966 Granholm and Siesjo 1969 Kjallquist *et al* 1969)

The present paper is an attempt to critically assess the effects of various anesthetics upon the energy state of the brain as reflected in the tissue concentrations of phosphocreatine lactate pyruvate ATP ADP and AMP Thus in all the experiments to be reported the body temperature and the arterial CO_2 and O_2 tensions were kept within narrow limits and autolytic changes were minimized by using optimal freezing and extraction techniques Further since measurements were made of also the blood and the CSF lactate and pyruvate concentrations intracellular lactate and pyruvate concentrations and lactate/pyruvate ratios could be derived Finally since intracellular acid base parameters were evaluated calculations of cytoplasmatic NADH/NAD ratios could be performed

Methods

The experiments were performed on male Wistar rats, weighing 300—400 g, which were allowed free access to water and to commercial rat pellets (San Bolagen, Malmo) All animals were tracheotomized, immobilizd with i p tubocurarine chloride (Tubocurarin Klorid, Vitrum),

measurement of the whole blood lactate and pyruvate concentrations

After that at least two sets of blood samples had been taken with an interval of 10—20 min 50—100 µl of cisternal cerebrospinal fluid were sampled by puncturing the exposed atlanto-

since this could lead to a faster fall in blood pressure

The CSF was usually analysed for the lactate and pyruvate concentrations but in some experiments the total CO_2 content was determined, using the micro diffusion method previously described (Siesjö 1962)

The brain tissue was stored at −85° to −90° C until it was subsequently analysed for the total CO_2 content (Ponten and Siesjö 1964) and for the phosphocreatine ATP ADP AMP lactate and pyruvate concentrations These metabolites were determined with the specific enzymatic techniques described by Hohorst *et al* (1959, *cf* also Schmahl *et al* 1965) However the tissue was extracted at −10° to −15° C, using frozen 3 M perchloric acid as described by Lowry *et al* (1964) After further disintegration of the tissue at 0° C, and a second extraction with 3 % perchloric acid, the combined supernatants were neutralized with 5 N KOH to pH 5 5 under electrometric pH control Measurements were performed at 340 mμ and each enzymatic curve was recorded on a Sargent SRL lin log recorder, attached to the Zeiss PMQ II spectrophotometer In order to increase sensitivity pyruvate, ADP and AMP were measured in 2 cm cuvettes However, with the freezing method employed the AMP concentrations were too low to allow accurate measurements Thus when a blank correction was applied for the AMP contamination of the NADH used (Lowry *et al* 1964 the net extinction changes obtained were usually lower than 0 015 Accordingly although the results showed that the AMP concentrations were very low, the differences between the groups must be interpreted with caution

The tissue contents of total CO_2 lactate, pyruvate, ATP, ADP, AMP and phosphocreatine have been given in mmoles/kg of wet tissue However, since also the CSF and the blood lactate and pyruvate concentrations were measured, intracellular concentrations (mmoles/kg of i c water) were calculated by applying corrections for a 3 % blood and a 12 % extracellular volume A similar correction was
ments (nitrous oxide, 7 % ether
differences from values measurec
Kaasik *et al* 1970 b) a CSF bic
present experimental groups

The intracellular pH was derived from the i c bicarbonate concentration and from the tissue CO_2 tension, the latter being calculated from the arterial CO_2 tension, (Pontén and Siesjo 1966 Brzezinski *et al* 1967) The cytoplasmatic NADH/NAD⁺ ratio was calculated from the pH_i and from the intracellular lactate/pyruvate ratio using a K′ value of 1 11 10 [11] (Williamson *et al* 1967, see also Granholm and Siesjo 1969 Kaasik *et al* 1970 a and b)

Administration of anesthetics The volatile anesthetics studied were nitrous oxide halothane (Halothan®, Hoechst) and diethyl ether (Aether ad narcosin Ph suec ed XI), and the barbiturates used included phenobarbital (Fenemal ACO), thiopental (Penthotalsodium® Abbott) and amobarbital (Isomyl® natrium Hassle) In addition in order to allow studies on conscious animals fentanyl citrate (Leptanal®, Leo) was used in a dose which induced marked analgesia

In all animals given volatile anesthetics, anesthesia was induced with divinyl ether (Vinydan®, Lundbeck) in a closed jar As soon as the animals were unresponsive to external stimuli (within 1—2 min) they were taken from the jar immediately tracheotomized and connected to the respirator Simultaneously
i p *Nitrous oxide* was used in a c
with oxygen *Cyclopropane* was p
individually calibrated
Lubeck respectively)
The gas flow through
dial setting used was
0 6 % for halothane and 4 and 7 % respectively for ether

Amobarbital was given i p in a dose of 100 mg/kg while *phenobarbital* was given i p in doses of 50 150 and 250 mg/kg respectively In all but the 50 mg/kg phenobarbital group the animals could be tracheotomized and operated upon 10—15 min after the injection In *the group given 50 mg/kg of phenobarbital anesthesia was induced with divinyl ether* and maintained with nitrous oxide until the operative procedures had been finished At that time nitrous oxide was withdrawn In the *thiopental* group the drug was slowly (6 min) given i v in a dose of 7 mg/kg during nitrous oxide anesthesia which was discontinued at the time of injection A similar procedure was followed for *fentanyl* which was given s c in two doses of 0 013 mg/kg with an interval of 25 min the nitrous oxide being withdrawn at the time of the first injection

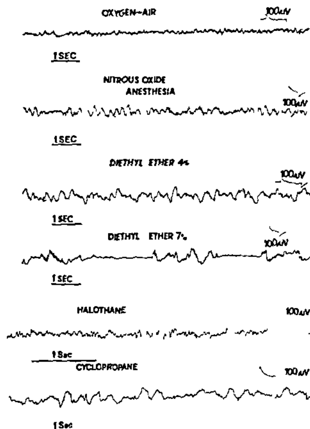


Fig 1 Representative EEG recordings from rats anesthetized with various volatile anesthetics (see Methods) The top recording (oxygen air) was obtained after that the nitrous oxide administration had been discontinued for 10 min

With all volatile anesthetics anesthesia was maintained for 30—50 min before CSF and brain tissue were sampled When anesthetic doses of phenobarbital and when amobarbital were used the total anesthetic periods were 60—100 min In the case of thiopentone CSF and brain tissue were sampled 12 min after the end of the injection of this shortlasting barbiturate

in control animals using two fronto-temporal brass screws secured into the skull bone, and a 2 channel grass EEG apparatus Fig 1 shows that even with the nitrous oxide or the halothane anesthesia there was some slowing of the EEG

few minutes The effect lasted for approximately 30 min Unconsciouness was not produced even if the dose was increase 4 times

Results

The effect of the various anesthetics on the body temperature and on the arterial blood pressure, as well as on the blood acid base parameters, are illustrated in Table I It can be seen that the maximal mean difference in rectal temperature between the groups was 0 6° C, and that the mean arterial blood pressure was 120 mm Hg or higher except in the halothane group (110 mm Hg) and in the group given phenobarbital in a dose of 250 mg/kg (90 mm Hg) No group had a mean hemoglobin concentration of less than 14 8 g/100 ml and the maximal difference in the mean CO_2 tension between the groups was 3 mm Hg (36 6—39 6) No animal included in any of the groups had an arterial oxygen tension of less than 85 mm Hg and the mean O_2 tensions varied between 105 and 125 mm Hg The tissue results reported below can thus be assumed to be uninfluenced by any obvious variations in the blood O_2 and CO_2 tensions in the hemoglobin concentration in the body temperature or in the mean arterial blood pressure

The lactate and pyruvate concentrations in arterial blood and in cisternal CSF have been given in Table II In the blood the mean lactate concentrations varied between 1 82 and 4 15 mmoles/kg Low values were found in the halothane and in the barbiturate groups and high values in the groups given diethyl ether (*cf* review by Ngai and Papper 1962) the lactate/pyruvate ratios varying in the same direction as the lactate concentrations The results did not suggest that the CSF concentrations varied passively with those of the blood Thus the low blood lactate concentrations in most of the barbiturate groups were accompanied by relatively high CSF concentrations and the variations in the lactate/pyruvate ratios of arterial blood were consistent with a marked stability of the CSF lactate/pyruvate ratios

Although the lactate concentration of arterial blood did not seem to determine the CSF lactate concentration it appeared to influence the whole blood base excess values Thus there was a fair correspondence between the lactate concentration and the base excess value the Fentanyl group and the 250 mg/kg phenobarbital group forming noteable exceptions (Fig 2)

TABLE I Rectal temperature, mean arterial blood pressure (MABP), hemoglobinconcentratic (Hb conc) and arterial acid base parameters in rats with various forms of anesthes (see text) Means ± s e

Exper group	Temp °C	MABP mm Hg	Hb conc g%	pCO_2 mm Hg	pH	Act HCO_3 mEq/l	Base excess mEq/l
Fentanyl n=6	36 9 ±0 2	170 ±10	15 7 ±0 7	38 6 ±1 4	7 365 ±0 010	21 3 ±1 0	−2 8 ±0 9
Nitrous oxide 70 % n=7	37 0 ±0 1	155 ±5	15 4 ±0 3	39 3 ±1 6	7 403 ±0 008	23 5 ±1 0	1 0 ±0 9
Halothane 0 6 % n=9	37 2 ±0 1	110 ±5	15 2 ±0 4	37 9 ±0 9	7 435 ±0 012	24 7 ±0 6	1 3 ±0 8
Diethyl ether 4 % n=9	37 0 ±0 1	125 ±5	15 4 ±0 3	38 1 ±0 6	7 401 ±0 008	22 7 ±0 4	−0 6 ±0 7
Diethyl ether 7 % n=14	37 1 ±0 1	120 ±5	14 8 ±0 4	39 6 ±0 5	7 359 ±0 013	21 4 ±0 5	−3 0 ±0 7
Cyclopropane 13 % n=10	36 7 ±0 1	135 ±10	14 8 ±0 4	38 9 ±1 1	7 413 ±0 016	24 1 ±0 8	−0 1 ±1 0
Phenobarb 50 mg/kg n=6	37 2 ±0 2	155 ±10	15 1 ±0 4	37 0 ±0 8	7 428 ±0 012	23 6 ±0 5	0 6 ±0 7
Phenobarb 150 mg kg n=6	37 2 ±0 2	140 ±10	15 3 ±0 2	39 6 ±1 1	7 421 ±0 015	24 8 ±0 5	1 1 ±0 7
Phenobarb 250 mg kg n=8	36 9 ±0 1	90 ±5	16 3 ±0 5	36 7 ±0 4	7 387 ±0 007	21 3 ±0 4	−2 6 ±0 5
Pentothal 7 mg kg n=8	37 3 ±0 1	120 ±5	14 9 ±0 3	37 1 ±1 5	7 446 ±0 018	24 6 ±0 8	1 6 ±0 8
Amobarb 100 mg kg n=5	36 8 ±0 2	160 ±15	15 2 ±0 3	36 6 ±1 8	7 456 ±0 016	25 1 ±0 9	2 2 ±0 9

Table III gives all the directly measured tissue metabolites There was a striking similarity in the concentrations of the labile phosphates Thus there was only a minor decrease in the phosphocreatine concentration in the Fentanyl group, and possibly slightly higher phosphocreatine concentrations in the phenobarbital animals The only other differences concerned the ADP concentration which seemed significantly decreased in the halothane group (see also amobarbital) when compared to *e g* the nitrous oxide animals

There were very small differences in the tissue lactate concentrations between the nonbarbiturate groups However there were significant decreases in the lactate concentrations in all groups given barbiturates when compared to *e g* the nitrous oxide animals The pyruvate concentration varied much less whence the differences in lactate implied a similar variation in the lactate/pyruvate ratio (see below)

Table IV gives the calculated intracellular parameters The calculations strengthened the impression given by Table III in showing decreases in the lactate concentrations, and in the lactate/pyruvate ratios in all groups injected with barbiturates The table makes it clear that in halothane anesthesia, which was associated with relatively low lactate concentrations and low lactate/pyruvate ratios in arterial blood

TABLE II Lactate and pyruvate concentrations (mmoles kg) in arterial blood and cisternal CSF in rats with various forms of anesthesia Means ± s e

Exper group	Arterial blood			CSF		
	La	Py	La/Py	La	Py	La/Py
Fentanyl n=[illegible]	2.63 ±0.13	0.187 ±0.018	14.7 ±1.3	2.72 ±0.11	0.193 ±0.012	14.2 ±0.6
N[illegible] oxide 70 % n=[illegible]	2.84 ±0.31	0.185 ±0.019	15.5 ±1.1	2.98 ±0.17	0.196 ±0.018	16.0 ±1.3
Halothane 0.6 % n=9	1.82 ±0.13	0.147 ±0.007	12.6 ±1.1	2.40 ±0.11	0.170 ±0.013	14.3 ±0.6
Diethyl ether 4 % n=9	3.40 ±0.39	0.207 ±0.028	16.7 ±1.4	2.88 ±0.15	0.154 ±0.006	18.7 ±0.8
Diethyl ether 7 % n=14	4.15 ±0.21	0.233 ±0.019	18.3 ±1.1	3.24 ±0.05	0.166 ±0.006	19.7 ±0.7
Cyclopropane 13 % n=10	2.36 ±0.56	0.213 ±0.035	10.4 ±1.5	2.76 ±0.17	0.195 ±0.017	14.4 ±0.9
Phenobarb 50 mg/kg n=6	2.22 ±0.21	0.238 ±0.016	9.4 ±0.7	2.93 ±0.18	0.183 ±0.009	16.1 ±0.8
Phenobarb 150 mg kg n=6	1.91 ±0.48	0.184 ±0.033	10.0 ±0.9	2.94 ±0.24	0.176 ±0.008	16.7 ±1.1
Phenobarb 250 mg/kg n=8	2.38 ±0.42	0.195 ±0.031	12.3 ±2.0	3.55 ±0.07	0.212 ±0.019	17.1 ±1.4
Pentothal 7 mg kg n=8	2.20 ±0.27	0.192 ±0.019	11.7 ±1.2	2.74 ±0.13	0.155 ±0.005	17.7 ±1.0
Amobarb 100 mg/kg n=5	2.02 ±0.37	0.243 ±0.019	8.1 ±1.1	3.83 ±0.56	0.229 ±0.030	17.7 ±2.7

and in CSF (se above), the intracellular parameters were similar to those obtained in the other nonbarbiturate groups It should also be pointed out that the blood and CSF lactacidosis seen with 7 % ether was absent in the intracellular space

Table IV shows that with the exception of the group given 4 % ether there was a striking similarity in pH'_i between the nonbarbiturate groups and a significant increase in pH'_i in all the barbiturate groups The calculated intracellular NADH/

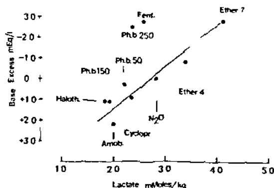


Fig 2 Relation between the whole blood lactate concentration and the calculated base excess values, in rats anesthetized with various anesthetics (cf Table I and II) The filled circles represent the mean values of 5—14 measurements

TABLE III The total CO_2 content (TCO_2) and the phosphocreatine, ATP, ADP, AMP, lactate and pyruvate concentrations of the supratentorial parts of rat brain, studied with various forms of anesthesia All values in mmoles/kg of wet tissue Means ± s e

Exper group	TCO_2	PCr	ATP	ADP	AMP	La	Py	La/Py
Fentanyl n=6	13 69 ±0 21	4 72 ±0 16	2 81 ±0 04	0 37 ±0 01	0 02 ±0 00	1 33 ±0 07	0 083 ±0 004	16 0 ±0 4
Nitrous oxide 70 % n=7	*13 71* ±0 36	*5 04* ±0 06	*2 80* ±0 04	*0 38* ±0 02	*0 02* ±0 01	*1 56* ±0 07	*0 098* ±0 005	*16 1* ±0 6
Halothane 0 6 % n=9	13 79 ±0 13	5 05 ±0 06	2 74 ±0 04	0 28 ±0 01	— —	1 34 ±0 05	0 084 ±0 004	16 1 ±0 6
Diethyl ether 4 % n=9	13 14 ±0 22	5 08 ±0 07	2 82 ±0 02	0 36 ±0 01	0 02 ±0 00	1 73 ±0 08	0 096 ±0 003	18 0 ±0 5
Diethyl ether 7 % n=14	14 13 ±0 21	±5 23 ±0 05	2 85 ±0 03	0 36 ±0 01	0 02 ±0 00	1 51 ±0 08	0 096 ±0 005	16 0 ±0 6
Cyclopropane 13 % n=10	13 61 ±0 30	5 04 ±0 09	2 72 ±0 05	0 35 ±0 01	0 03 ±0 00	1 51 ±0 09	0 096 ±0 005	15 8 ±0 7
Phenobarb 50 mg/kg n=6	13 96 ±0 23	5 21 ±0 07	2 84 ±0 07	0 36 ±0 02	0 01 ±0 00	±1 11 ±0 07	0 083 ±0 05	13 4 ±0 4
Phenobarb 150 mg/kg n=6	14 97 ±0 34	5 38 ±0 15	2 82 ±0 06	0 34 ±0 02	0 01 ±0 00	0 97 ±0 12	0 078 ±0 007	12 2 ±0 7
Phenobarb 250 mg/kg n=8	14 91 ±0 23	5 30 ±0 05	2 81 ±0 02	0 35 ±0 01	0 02 ±0 00	0 88 ±0 05	0 078 ±0 008	11 5 ±0 6
Pentothal 7 mg/kg n=8	14 53 ±0 19	5 15 ±0 10	2 81 ±0 04	0 35 ±0 02	0 03 ±0 01	1 06 ±0 03	0 071 ±0 003	15 3 ±0 8
Amobarb 100 mg/kg n=5	14 62 ±0 40	5 01 ±0 08	2 75 ±0 05	0 30 ±0 02	0 01 ±0 00	1 02 ±0 09	0 079 ±0 002	12 9 ±1 0

)NAD⁺ ratios by necessity showed some scatter but there seemed to be very small differences between the nonbarbiturate groups, and a significant lowering in barbiturate anesthesia (see phenobarbital 150 and 250 mg/kg, and amobarbital) There was a very good inverse relationship between the intracellular lactate concentration, and the calculated intracellular pH′, indicating that the same mechanisms which are responsible for the variations in the lactate concentrations also to some extent determine the pH_i (see Fig 3)

The present results make it possible to draw some conclusions regarding the distribution of lactate and pyruvate between the extra- and intracellular spaces Thus, it has been pointed out that since lactic acid and pyruvic acid are weak acids they may be distributed across the cell membranes according to the pH gradient ("non-ionic diffusion" se Hohorst *et al* 1959) If this is true the following relationship should hold

$$\frac{(HCO_3^-)_i}{(HCO_3^-)_{CSF}} = \frac{(La^-)_i}{(La^-)_{CSF}} = \frac{(Py^-)_i}{(Py^-)_{CSF}}$$

(see Granholm *et al* 1968) However Fig 4 shows that although the lactate and pyruvate distributions were very similar, they deviated markedly from the bicarbonate

TABLE IV.

Exper group	ptCO₂	HCO₃⁻ i c w	pHᵢ	La i c w	Py i c w	La/Py	NADH/NAD+
Fentanyl	45 0	13 39	7 102	1 41	0 081	17 4	2 45
n=6	±1 3	±0 32	±0 006	±0 10	±0 005	±0 7	±0 12
Nitrous oxide 70 %	45 6	13 24	7 089	1 77	0 106	16 9	2 32
n=7	±1 5	±0 43	±0 017	±0 08	±0 007	±0 9	±0 17
Halothane 0 6 %	44 4	13 36	7 100	1 57	0 099	16 2	2 27
n=9	±1 0	±0.20	±0 010	±0 08	±0 006	±0 6	±0 08
Diethyl ether 4 %	44 5	12 40	7 067	2 01	0 115	17 4	2 31
n=9	±0 6	±0 33	±0 013	±0 13	±0 005	±0 7	±0 11
Diethyl ether 7 %	45,9	13 86	7 102	1 59	0 111	14 4	2 04
n=14	±0 5	±0 32	±0 010	±0 14	±0 008	±0 6	±0 10
Cyclopropane 13 %	45 3	13 69	7 092	1 67	0 096	16 1	2 22
n=10	±1 1	±0 40	±0 014	±0 14	±0 005	±1 5	±0 26
Phenobarb 50 mg/kg	43 5	13 82	7 125	1 08	0 084	12 9	1 92
n=6	±0 7	±0 33	±0 007	±0 10	±0 007	±0 5	±0 09
Phenobarb 150 mg/kg	45 4	15 20	7 143	0 87	0 080	10 7	1 66
n=6	±1 0	±0 53	±0 010	±0 18	±0 010	±1 0	±0 18
Phenobarb 250 mg/kg	42 7	15 26	7.173	0 33	0 046	7 1	1 19
n=8	±0 4	±0 34	±0 008	±0 06	±0 004	±0 9	±0 14
Pentothal 7 mg/kg	43 6	14 68	7 166	1 04	0 076	13 7	2 06
n=8	±1 4	±0 31	±0 013	±0 06	±0 002	±0 8	±0 07
Amobarb 100 mg/kg	43 1	14 69	7 155	0 78	0 071	10 9	1 74
n=5	±1 7	±0 57	±0 017	±0 08	±0 005	±0 6	±0 11

distributions in most of the present groups A striking dissociation was seen with e g 4 % ether and with deep barbiturate anesthesia It seems safe to conclude that the distribution of lactate and pyruvate between the extra- and intracellular spaces of the brain is governed by other and more complex factors than the pH gradient

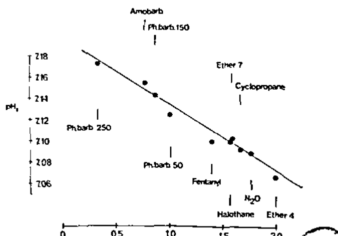


Fig 3 Relation between the derived intracellular lactate concentration and the calculated intracellular pH with various forms of anesthesia The filled circles represent mean values from 5—14 measurements Note linear relation between lactate concentration and pH ᵢ

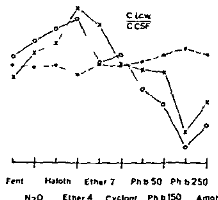


Fig 4 Comparison between the intracellular water/CSF concentration ratios for lactate pyruvate and bicarbonate in rats under various forms of anesthesia (see Table I—IV) Each symbol represents the mean of 5—14 expts Note that lactate and pyruvate are not distributed as is bicarbonate between intracellular water and CSF

Discussion

The present study of anesthetic effects on the extra- and intracellular lactate pyruvate and bicarbonate concentrations, and on the tissue concentrations of phosphocreatine ATP ADP and AMP in the rat brain, offers the advantage that all animals were subjected to the same operative procedures and had similar arterial CO_2 tensions, and in that accidental variations in body temperature and in arterial O_2 tension could be excluded Also, since anesthetic periods of 35—100 min were used after a preliminary induction with divinyl ether it can be assumed that the tissue substrates measured were representative of the anesthetic under study except in the case of thiopental (anesthetic time 12 min) However since the CSF composition may take a longer time to approach that of a steady state the CSF changes observed should be interpreted with some caution

The main results of the present study concern the tissue concentrations of labile phosphates and of lactate as well as the calculated intracellular pH' values and the NADH/NAD ratios With the exception of an unexplained decrease in the ADP concentration in the halothane group, there was a marked constancy in the tissue concentrations of ATP ADP and AMP in all animals studied There was also a very small variation in the phosphocreatine concentrations in all anesthetized animals Thus since the slightly lower phosphocreatine concentration in the conscious group (Fentanyl) may be assumed to be due to a breakdown caused by excitation during the freezing (see below) the results lend no support to the view that anesthesia by itself leads to an increase in the phosphocreatine concentration of the brain. This conclusion is supported by unpublished results from the laboratory which show that the phosphocreatine concentration varies with the intracellular hydrogen ion concentration Thus these results make it probable that the slight increases in the phosphocreatine concentration in most of the barbiturate groups were due to the alkalosis, and not to the depth of the anesthesia

There were no obvious differences in the tissue lactate concentration between the nonbarbiturate groups and thus no indication that the lactate concentration varied with the depth of the anesthesia In all barbiturate groups however there was a lowering of the lactate concentration a finding which was supported by the derived intracellular concentrations Thus the results indicate that barbiturates have a specific effect on the tissue lactate concentrations A corresponding effect was seen on the calculated intracellular pH in that almost identical values were obtained in the nonbarbiturate groups and in that all barbiturate groups showed an increase in pH′, which appeared to be dose dependent Finally the calculated NADH/NAD ratio did not seem to be affected by the depth of anesthesia in the nonbarbiturate groups but there were decreases in the animals anesthetized with barbiturates particularly in those which had been given high doses

Taken together the present results indicate that anesthesia with volatile anesthetics does not appreciably shift the balance between energy yielding and energy consuming reactions but that barbiturates have a larger inhibitory influence on reactions consuming energy than on oxidative reactions We could thus tentatively conclude that barbiturates induce a high energy state in the brain characterized by high levels of phosphocreatine low levels of lactate a reduced NADH/NAD ratio and an increased intracellular pH′ Before some details of these results are discussed a few methodological points must be brought up for discussion

Methods and assumptions It is generally agreed that even very shortlasting hypoxia leads to significant changes in *e g* the phosphocreatine AMP and lactate concentrations of the brain and that accordingly great care must be taken to avoid hypoxia during the fixation of the tissue It is thus unfortunate that so many studies of labile substrates in the brain have been carried out with immersion of the head or of the whole animal in liquid nitrogen or with decapitation of the animal before the freezing of the tissue All such procedures will lead to hypoxemia and to changes in the labile substrates of the brain before the tissue has been frozen (*cf* Granholm *et al* 1968) and these errors will be slight or insignificant only if very small animals are used (see references to work from Lowry s laboratory) In animals larger than young mice a different procedure must be used The procedure described by Kerr 1935) implies that the animal is protected against hypoxia while the head is being frozen and has the advantage that an adequate blood pressure and a constant ventilation are secured until the medullary centers have been reached by the freezing front (Ponten 1966 Granholm *et al* 1968) Thus the finding that this freezing technique when used in 300—400 g rats in conjunction with the extraction technique of Lowry *et al* (1964) gives phosphocreatine and AMP values similar to those obtained on anesthetized 10 g mice indicates that the size of the animal should be no obstacle when studying labile substrates in the brain

Although it is indicated that autolytic changes during the fixation of the tissue are minimized when very small animals are used it must be kept in mind that the substrate levels reported for unanesthetized animals may be influenced by brief hypoxia during the fixation of the tissue and such changes may be exaggerated by the ex

citation caused by the freezing. Thus the high phosphocreatine and the low AMP values obtained in the present study with such light levels of anesthesia as those obtained with 70 % nitrous oxide or with 0.6 % halothane (cf. Wollman et al. 1967) may be due to the protective effect of the anesthetic against excitation. This conclusion is strengthened by the slight lowering of phosphocreatine concentration seen in the group injected with Fentanyl since these animals although analgetic react forcibly to e.g. sound stimuli. We thus want to emphasize that if unanesthetized animals are found to have a lower phosphocreatine concentration than anesthetized ones this does not necessarily reflect the true *in vivo* phosphocreatine levels but may merely indicate that supravital autolytic changes are speeded up by the shock of the freezing procedure when no anesthetic is used.

The fact that most previous studies of substrate levels in the brain have been carried out without control of physiological parameters such as temperature, blood pressure and arterial O_2 and CO_2 tensions makes it difficult to interprete e.g. the lactate changes reported. It should be recalled in this context that whereas normocapnic rats under nitrous oxide anesthesia (pCO_2 about 35 mm Hg) have tissue lactate concentrations of 1.3—1.5 mmoles/kg, an increase in the CO_2 tension to 50—60 mm Hg or higher is associated with a fall in lactate to 1.0—1.1 mmoles/kg while a decrease in the arterial pCO_2 to about 25 mm Hg raises the lactate concentrations to more than 2 mmoles/kg (Kjällquist et al. 1969). It should thus be apparent that if the anesthetic under study gives rise to hyperventilation or to CO_2 retention, any changes observed in the lactate (or pyruvate) concentrations may be due to changes in the CO_2 tension and are not necessarily caused by the anesthetic state.

The effect of anesthetics on labile phosphates and on lactate and pyruvate concentrations. Provided that we may assume that the cytoplasmatic NADH/NAD ratio calculated from the present results can be used to indicate also the redox state of the mitochondria, we must conclude that the present experiments have failed to indicate an interference of anesthetics with electron transport or energy transfer reactions in the brain, at least under normal unstimulated *in vivo* conditions (see introduction). The results make it clear that anesthetics which inhibit energy yielding reactions in isolated mitochondrial systems (barbiturates and particularly amobarbital) apparently fail to do so *in vivo*. It remains to be studied if the anesthetics used have different effects on the energy metabolism of the stimulated tissue. However, since the present experiments included both conscious animals and animals in general anesthesia, any such effect must evidently be looked for under conditions of a pathological increase in the tissue metabolism.

Our results differ from those of others in two respect. Thus, we have been unable to detect any larger differences in e.g. the phosphocreatine concentrations between animals under light or deep anesthesia. Further, provided the Fentanyl group can be taken to represent the conscious state, the experiments showed that there was no decrease in the tissue lactate concentrations with volatile anesthetics. Thus our results indicate that even a very superficial anesthesia is accompanied by optimal

phosphocreatine and AMP concentrations, and, further, that the decrease in the tissue lactate (and pyruvate) concentrations is specific for barbiturates

The effect of anesthetics on the calculated intracellular parameters There was a striking similarity in the pH'ᵢ values and in the NADH/NAD ratios derived for the intracellular space in the groups exposed to volatile anesthetics with the possible exception of deep ether anesthesia. Thus, in the only group with an apparent increase in the lactate/pyruvate ratio (4 % ether) the pH'ᵢ was sufficiently acid to yield a "normal NADH/NAD ratio The effect of barbiturates upon the intra cellular lactate and pyruvate concentrations appeared to be dose dependent, and it should be recalled that the low lactate concentration and the low NADH/NAD⁺ ratio in the 250 mg/kg phenobarbital group were consistent with relatively high blood and CSF lactate concentrations

Supported by the Swedish Medical Research Council (Project No B70-14X 263 06 and B70 40X 2179 02) by the Swedish Bank Tercentary Fund and by PHS Research Grant No 1 ROI NB07838 01 from NIH

References

BRZEZINSKI J A KJALLQUIST and B K SIESJÖ Mean carbon dioxide tension in the brain after carbonic anhydrase inhibition *J Physiol* (Lond) 1967 *188* 13—23

CHANCE B, Effects of anesthesia on metabolism and cellular functions *Pharmcol Rev* 1965 *17* 191—195

CHANCE B P COHEN F JOBSIS and B SCHOENER Intracellular oxidation reduction states in vivo *Science* 1962 *137* 499—508

CHANCE B and G HOLLUNGER Inhibition of electron and energy transfer in mitochondria. I Effects of Amytal thiopental rotenone progresterone, and methylene glycol *J biol Chem* 1963 *238* 418—431

ERNSTER L G DALLNER and G F AZZONE Differential effects of rotenone and amytal on mitochondrial electron and energy transfer *J biol Chem* 1963 *238* 1124—1131

GATFIELD P D O H LOWRY D W SCHULZ and J V PASSONNEAU Regional energy reserves in mouse brain and changes with ischaemia and anesthesia *J Neurochem* 1966 *13* 185—195

GOLDBERG N D J V PASSONNEAU and O H LOWRY Effects of changes in brain metabolism on the levels of citric acid cycle intermediates *J biol Chem* 1966 *211* 3997—4003

GRANHOLM L [illegible]
cerebrospinal f [illegible]
phosphocreatin [illegible]

GRANHOLM L a [illegible]
spinal fluid l [illegible]
phosphocreatir [illegible]
75 257—266

HOHORST H J F H KREUTZ and T BUCHER Über Metabolitgehalte und Metabolit Konzentrationen in der Leber der Ratte *Biochem Z* 1959 *332* 18—46

KAASIK A E L NILSSON and B K SIESJO The effect of asphyxia upon the lactate pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF and upon the tissue concentrations of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970 a *78* 433—447

KAASIK A E L NILSSON and B K SIESJO The effect of arterial hypotension upon the lactate pyruvate and bicarbonate concentrations of brain tissue and cisternal CSF and upon the tissue concentration of phosphocreatine and adenine nucleotides in anesthetized rats *Acta physiol scand* 1970 b *78* 448—458

KERR S E Studies of the phosphorus compounds of brain I Phosphocreatine *J biol Chem* 1935 *110* 625—635

KJALLQUIST A M NARDINI and B K SIESJO The regulation of extra and intracellular acid base parameters in the rat brain during hyper and hypocapnia. *Acta physiol scand* 1969 *76* 485—494

LEUSEN, I and G DEMEESTER, Lactate and pyruvate in the brain of rats during hyperventilation *Arch int Phys Bioch* 1966 *74* 25—34
LOWRY, O H, J V PASSONNEAU, F X HASSELBERGER and D W SCHULZ, Effect of ischemia on known substrates and cofactors of the glucolytic pathway in brain *J biol Chem* 1964 *239* 18—30
MAYMAN, C I, P D GATFIELD and B McL BRECKENRIDGE, The glucose content of brain in anesthesia *J Neurochem* 1964 *11* 483—487
McILWAIN, H, *Biochemistry and the Central Nervous System*, J & A. Churchill Ltd, London 1966 p 43 and 338—345
NGAI S H and E M PAPPER, *Metabolic effects of anesthesia* Charles C Thomas, Springfield, Illinois 1962
PONTEN, U, Acid base changes in rat brain tissue during acute respiratory acidosis and baseosis *Acta physiol scand* 1966 *68* 152—163
PONTEN, U and B K. SIESJO, A method for the determination of the total carbon dioxide content of frozen tissues *Acta physiol scand* 1964 *60* 297—308
PONTEN U and B K. SIESJO, Gradients of CO_2 tension in the brain *Acta physiol scand* 1966 *67* 129—140
QUASTEL J H Effects of anesthetics depressants and tranquilizers on brain metabolism. In *Neurochemistry the Chemistry of Brain and Nerve*, ed by K. A Elliot J H Page and J H Quastel, 2nd ed, Charles C Thomas, Springfield, 1962 pp 790—812
RICHTER, D and R M C DAWSON Brain metabolism in emotional excitement and in sleep *Amer J Physiol* 1948 *154* 73—79
SCHMAHL F W E BETZ H TALKE and H J HOHORST, Energiereiche Phosphate und Metabolite des Energiestoffwechsels in der grosshirnrinde der Katze *Biochem Z* 1965 *342* 518—531
SIESJO B K The solubility of carbon dioxide in cerebral cortical tissue of cats With a note on the solubility of carbon dioxide in water, 0 16 M NaCl and cerebrospinal fluid *Acta physiol scand* 1962 *55* 325—341
SIGGAARD-ANDERSEN O Blood acid base alignment nomogram *Scand J clin Lab Invest* 1963 *15* 211—217
STONE W E, The effects of anesthetics and of convulsants on the lactic acid content of the brain *Biochem J* 1938 *32* 1908—1918
WILLIAMSON D H PATRICIA LUND and H A KREBS The redox state of free nicotinamide adenine dinucleotide in the cytoplasma and mitochondria of rat liver *Biochem J* 1967 *103* 514—527
WOLLMAN H S CRAIGHEAD ALEXANDER and P J COHEN Cerebral circulation and metabolism in anesthetized man In *Neurologic Considerations*, ed by M H Harmel Blackwell Sci Publ Oxford 3/1967 1—15

Acta physiol. scand. 1970. 80. 249—283

From the Institute of Pharmacology and Toxicology, Royal Veterinary and Agricultural University, Copenhagen

Acetylation of P-Aminohippuric Acid in the Kidney. Renal Clearance of P-Aminohippuric Acid and N^4-Acetylated P-Aminohippuric Acid in Pigs

By

N. GYRD-HANSEN and FOLKE RASMUSSEN

Received 26 February 1970

Abstract

GYRD-HANSEN, N. and F. RASMUSSEN. *Acetylation of p-aminohippuric acid in the kidney. Renal clearance of p-aminohippuric acid and N^4-acetylated p-aminohippuric acid in pigs.* Acta physiol. scand. 1970. *80*. 249—253.

[illegible]

were 91 ± 1.4 (S.E.M.) and 84 ± 2.1 respectively. The ratio between clearance of N^4 acetylated PAH and PAH was found to be 1.03 ± 0.07 and between clearance for PAH_{total} and PAH 1.01 ± 0.02.

Renal clearance of p-aminohippuric acid (PAH) can be used for determination of the effective renal plasma flow ($RPF_{effective}$) only if metabolism of PAH does not take place to any great extent during passage through the kidneys. However, Rennick, Hamilton and Evans (1961) and Setchell and Blanch (1961) showed that PAH is conjugated *in vitro* by kidney slices in many animal species. The conjugated derivative is probably N^4-acetylated PAH, and Nielsen, Maaske and Booth (1966) stated that PAH cannot be used for determining $RPF_{effective}$ in pigs because the kidney acetylates PAH too rapidly. The aim of the present study was to determine the extent of this acetylation and to assess its significance for determination of $RPF_{effective}$ in pigs.

Material and methods

The experiments were carried out on 17 female and 3 (no. 1, 2 and 3) castrated male Da[illegible] race pigs weighing 21—124 kg. The animals were fed during the observation period [illegible] mal fodder mixture as described by Gyrd-Hansen (1968) and they were allowed free a[illegible]

was given by continuons intravenons infusion

The concentration of PAH was determined in urine and plasma by the method described by Bratton and Marshall (1939) for sulphonamide determination PAH_{total} (PAH + N^4 acetylated PAH) was estimated on the same samples after hydrolysis with hydrochloric acid All analyses were done in duplicate The concentration of N^4 acetylated PAH was determined as the difference between PAH_{total} and PAH PAH and PAH_{total} were estimated with a standard deviation of 1 4 % in both plasma and urine PAH and acetylated PAH could not be detected in blood plasma and urine sampled before the start of the experiment

The extent of acetylation or de acetylation in the kidney was calculated from the concentrations of PAH and N^4 acetylated PAH in plasma from a renalis and v renalis and in urine

The following symbols were used in the calculations —

A_{PAH} = Concentration of PAH in plasma from a renalis
$A_{a\text{-}PAH}$ = Concentration of N^4-acetylated PAH in plasma from a renalis
V_{PAH} = Concentration of PAH in plasma from v renalis
$V_{a\ PAH}$ = Concentration of N^4 acetylated PAH in plasma from v renalis
U_{PAH} = Concentration of PAH in urine
$U_{a\ PAH}$ = Concentration of N^4 acetylated PAH in urine

The ratio $U_{a\ PAH}/U_{PAH}$ is designated k
D = Urine flow
F = Renal plasma flow

The total amount of PAH plus N^4 acetylated PAH excreted in the urine must correspond to the amount of PAH plus N^4 acetylated PAH removed by the passage of blood through the kidney therefore

(1) $(U_{PAH} + U_{a\ PAH})D = (A_{PAH} - V_{PAH} + A_{a\ PAH} - V_{a\ PAH})F$

Substitution of $k \times U_{PAH}$ instead of $U_{a\ PAH}$ gives the following

(2) $(U_{PAH} + k \times U_{PAH})D = (A_{PAH} - V_{PAH} + A_{a\ PAH} - V_{a\ PAH})F$

(3) $U_{PAH} = \frac{(A_{PAH} - V_{PAH} + A_{a\ PAH} - V_{a\ PAH})F}{(1+k)D}$

From (1) and (3) the following is obtained

(4) $U_{a\ PAH} = \frac{F(A_{PAH} - V_{PAH} + A_{a\ PAH} - V_{a\ PAH})}{D} - \frac{F(A_{PAH} - V_{PAH} + A_{a\ PAH} - V_{a\ PAH})}{(1+k)D}$

The acetylation percentage is calculated as percentage of the amount of PAH delivered to the kidney by the renal artery

(5) Acetylation % $= \frac{U_{a\ PAH} \times D - (A_{a\ PAH} - V_{a\ PAH})F}{A_{PAH} \times F} \times 100$

The placing of (4) in (5) and reduction gives

Acetylation % $= \frac{k \times (A_{PAH} - V_{PAH}) - (A_{a\ PAH} - V_{a\ PAH})}{A_{PAH} \times (1+k)} \times 100$

A negative acetylation precentage indicates de acetylation

Analysis of variance and statistical calculations of standard deviation (S D) standard error of the mean (S E M) and p were determined by standard techniques (Freund 1967)

Results

Acetylation and de-acetylation

9 expts , each with 5 observations, were carried out in 9 pigs in order to determine the degree of acetylation of PAH in the kidney In Table I the mean values of the 5 observations for each animal are shown It is seen that the acetylation percentage

TABLE I Acetylation and de-acetylation in the kidney*

Animal no	Renal artery		Renal vein		Urine	Acetylated in the kidney as percentage of the amount of PAH delivered to the kidney
	PAH µg/ml	N⁴ a PAH µg/ml	PAH µg/ml	N⁴ a PAH µg/ml	N⁴ a PAH/PAH	
1	45	11	2.0	1.9	0.35	10.1
2	40	13	3.3	2.7	0.39	6.3
3	23	8.5	0.9	0.9	0.44	4.0
4	70	33	5.6	2.6	0.21	—21.1
5	43	18	7.3	4.1	0.29	— 6.3
6	74	14	7.5	4.0	0.22	4.7
7	13	3.9	1.8	0.5	0.33	1.5
8	94	10	5.5	1.5	0.09	— 2.1
9	110	18	13.8	2.4	0.19	2.0
Mean ±S.E.M.						— 0.1±3.1

* The figures are average values of 5 observations

varies between + 10.1 and —21.1, with an average of —0.1 %. This means that acetylation of PAH was found in 6 of the pigs, while there was de-acetylation of N⁴-acetylated PAH in the last 3 ones. By analysis of variance was shown that there was a significant difference among the animals ($p<0.001$) while there were no significant differences within the periods ($p>0.50$). This means that the analytical error itself can not explain the variations in acetylation percentage.

Extraction percentages

The extraction percentage $\left(\frac{A_{conc}-V_{conc}}{A_{conc}} \times 100\right)$ for PAH and N⁴-acetylated PAH were calculated from the concentration of PAH and N⁴-acetylated PAH in plasma from a. renalis and v. renalis (Table I). The arterial concentrations varied between 13 and 110 µg PAH/ml and 4—33 µg N⁴ acetylated PAH/ml, and the average extraction percentages were 91 per cent for PAH and 84 cent per for N⁴ acetylated PAH (Table II). The arterial blood pressure was between 90/75 and 165/105 mm Hg during the experiments.

Clearance of PAH and N⁴-acetylated PAH

The concentration of N⁴ acetylated PAH in plasma was in average 28 per cent of the concentration of PAH_{total} (Table III). The ratio between the clearance of

TABLE II Renal extraction of p-aminohippuric acid and N⁴ acetylated p-aminohippuric acid

Extraction percentage of	Animal no									Mean ±S.E.M.
	1	2	3	4	5	6	7	8	9	
PAH	95	92	96	92	83	90	86	94	88	91±1.4
N⁴ a PAH	83	80	89	92	77	72	87	86	87	84±2.1

TABLE III Renal clearance of p-aminohippuric acid and N^4 acetylated p-aminohippuric acid

Animal no	Body weight kg	Concentrations in plasma			
		PAH μg/ml	N^4 a PAH μg/ml	PAH_{total} μg/ml	% N^4 a PAH in plasma
1—9	47—95	13—109	4—32	17—128	10—33
10—20	21—124	12—28	5—17	17—41	20—44
Mean ± S E M					28 ± 1 7

N^4 acetylated PAH and PAH varied between 0 40 and 2 09 mean 1 08 ± 0 07 (S E M) The ratio between the clearance of PAH_{total} and PAH varied from 0 79 to 1 23, with a mean of 1 01 ± 0 02 (S E M)

Discussion

It was shown by the work of Setchell and Blanch (1961) that kidney tissue from pigs acetylates PAH *in vitro* In the present study, acetylation of PAH was demonstrated *in vivo* in the kidneys of 6 pigs, while in 3 animals there was de acetylation of N^4 acetylated PAH in the kidneys The percentage acetylation and de acetylation in the kidneys involves a corresponding error in the PAH clearance and consequently the $RPF_{effective}$ This means that in six cases the clearance of PAH and $RPF_{effective}$ would be determined 15—10 per cent lower and in three cases 2—21 per cent higher than if metabolism of PAH and N^4 acetylated PAH did not take place in the kidneys The error caused by metabolism of PAH and N^4 acetylated PAH in the kidneys can be avoided if PAH_{total} is used for calculation of PAH clearance and $RPF_{effective}$

The large variations in the ratio between clearance of N^4 acetylated PAH and PAH (Table III) may partly be due to acetylation and de acetylation in the kidney and partly to the low concentration of N^4 acetylated PAH in plasma estimated as the difference between PAH_{total} and PAH A comparison between the clearance of PAH_{total} and PAH (Table III) shows that the mean of the ratio between $C_{PAH\,total}$ and C_{PAH} is 1 01 ± 0 02 (S E M) with variations from 0 79 to 1 23 The analytical error on these clearance ratios is 2 8 % (S D) and is obviously small in comparison with the variations in clearance ratios This means that an acetylation and de acetylation has taken place in the kidneys

Whether use of PAH clearance is unsuitable for calculating $RPF_{effective}$ *in pigs*, as reported by Nielsen et al (1966) depends partly on the number of animals and on the purpose of the $RPF_{effective}$ determination If the number of animals is small PAH_{total} is preferable in determining absolute $RPF_{effective}$, since in individual determinations there could be an error of up to 20 per cent if PAH were used (*last column of Table III*) However, if the mean value for a large number of animals is required clearance of PAH and PAH_{total} will give identical $RPF_{effective}$ values since the average ratio between clearance of PAH_{total} and PAH is 1 01

Clearance of			Clearance ratio	
PAH ml/min/10 kg	N^4-a-PAH ml/min/10 kg	PAH_{total} ml/min/10 kg	N^4-a-PAH/PAH	PAH_{total}/PAH
16—43	17—44	18—34	0 40—1 44	0 79—1 12
53—106	42—161	58—105	0 66—2 09	0 90—1 23
			1 08 ± 0 07	1 01 ± 0 02

References

BRATTON, A. C. and E. K. MARSHALL, A new coupling component for sulfanilamide determination. *J biol Chem* 1939, *128* 537—550

[illegible]

SETCHELL, B. P. and E. BLANCH, Conjugation of p-aminohippurate by the kidney and effective renal plasma flow. *Nature* (Lond.) 1961, *189*, 230—231

Acta physiol. scand. 1970. 80. 254—268

From the Institute of Zoophysiology, University of Uppsala, Sweden

The Circadian Fluctuations of the Amount of Free Phosphate and of the Activity of Acid Phosphatase in the Kidneys of Mice and the Effect of UV Radiation upon this Rhythm

By

PER ERIC LINDAHL and JÓSEF SUROWIAK*

Received 26 February 1970

Abstract

LINDAHL, P E and J SUROWIAK *The circadian fluctuations of the amount of free phosphate and of the activity of acid phosphatase in the kidneys of mice and the effect of UV radiation upon this rhythm* Acta physiol scand 1970 80 254—268

the day indicating the existence of
males and in females and exhibits
kidney homogenate varies in an
changes both in the enzyme activity and in the content of inorganic phosphate In irradiated animals the new maxima in the activity of acid phosphatase were generally accompanied by maxima in the amount of P_i It is supposed that the end products of the reactions in the skin formed under the influence of UV radiation and associated with the degradation of phospholipids are the main factors responsible for the observed changes in the kidney

Almost all physiological processes occurring in animal organisms show some rhythmic variability of their intensity related either to the diurnal sequence day/night or to changes of the seasons of the year Experimental achievements in this field have been described in detail by Sollberger (1965) and Aschoff (1965) while a recent paper by Surowiak (1969) has provided some supplementary data

The activity of acid phosphatase shows certain rhythmic fluctuations depending on the seasons of the year the intensity of light or the length of day and night Quantitative alterations in the activity of this enzyme in the hypothalamus pituitary system of the sparrow as a consequence of stimulation by light were reported by Kobayashi and Farmer (1960) Similar changes in the pituitary, thyroid and adrenal glands of mice kept for prolonged periods of time in darkness or light as well as

[1] Abbreviation P_i=inorganic phosphate
* Present adress Departmetn of Animal Physiology Jagiellonian University Krakow, Poland

after irradiation of the animals with a very intense white light, were observed by Surowiak and Tilgner (1968)

Finally, Surowiak (1969) demonstrated marked daily variations in the activity of this enzyme in the hypothalamus and the pituitary, thyroid and adrenal glands of mice and showed that this rhythm is altered by exposure to UV or X radiation

The activity of acid phosphatase shows daily changes in the neurohypophysis the gland releasing the antidiuretic hormone It seems therefore of interest to study whether similar changes in the activity of this phosphatase occur in the kidney, the physiological activity of which is partly controlled by this hormone, and whether these changes are correlated in time with those in the neurohypophysis It was the aim of the present study to investigate by means of chemical analysis the variation in P_i and in the activity of acid phosphatase during the day and the influence of UV radiation upon these variations in the kidney of the mouse

Material and methods

In each experiment 126 inbred albino mice aged 8 to 12 weeks were used The sex ratio of the animals was 1 1 The mean weight of the females was 23 6 ± 1 88 g and that of the males 27 83 ± 2 66 g The animals were reared in a windowless room in artificial light between 08 00 and 20 00 After 10 days of adoption of the animals to this light rhythm the following experiments were carried out

In the control group consisting of 28 males and 28 females the activity of acid phosphatase in the kidney homogenate and the amount of P_i were measured every 6 hrs starting from 12 00 Seven males and seven females were used for every measurement (at 12 00 18 00 24 00 and 06 00)

In the experimental group consisting of 35 males and 35 females identical determinations were carried out with animals previously treated with UV radiation between 10 00 and 11 00 Hence the exposure terminated 1 hr before the first determination of the enzymatic activity

A quartz burner (S-500 Original Hanau) was the source of UV radiation A 6-cm layer of water in a quartz vessel was used as an infrared filter The distance between animal and burner was 30 cm The intensity of the UV radiation of the single total dose was approximately 80 000 erg/sec/cm² for 1 hr The animals were killed by cervical dislocation and decapitation The excised kidneys were weighed at 0° C in 0 25 M sucrose solution containing 0 05 M acetate buffer pH 5 0 After threefold washing of the kidney in the sucrose solution the organ was homogenized with 3 0 ml of this solution (Gianetto and de Duve 1955 Karlsson 1969) and the homogenate was centrifuged at 800 *g* for 20 min at 4° C Under these conditions the homogenate separates into two fractions the supernatant quite limpid and bright yellow in colour or slightly red after exposure to UV radiation and the sediment (Weissman and Thomas 1963 The sediment was re suspe

its two fractions the amount of P

β glycerophosphate according to

calculation of the enzyme activity

d the activities of the acid phosphatase

and de Duve 1956 Wattiaux *et al* 1956) of the acid phosphatase in the homogenate was shown to occur only in the sediment fraction Activation was attained in these experiments by incubating 2 0 ml of homogenate for 45 min at 37° C with 1 0 ml of a 0 1 % solution of Triton X 100 in 0 05 M acetate buffer pH 5 0 de Duve Wattiaux and Wibo 1962 Sawant *et al* 1964 Karlsson 1969) Hence the activity of acid phosphatase in the supernatant was measured directly without previous incubation with Triton

To sum up the activity of acid phosphatase and the amount of P_i were analyzed in homogenates and in supernatant and sediment fractions obtained from control and experimental animals killed at 12 00 18 00 24 00 and 06 00 and from experimental animals killed at 12 00 on the following day All the results were evaluated by employing Student's *t* test to test the significance of the differences between the mean values found

Acta physiol. scand. 1970. 80. 254—268

From the Institute of Zoophysiology, University of Uppsala Sweden

The Circadian Fluctuations of the Amount of Free Phosphate and of the Activity of Acid Phosphatase in the Kidneys of Mice and the Effect of UV Radiation upon this Rhythm

By

Per Eric Lindahl and Józef Surowiak*

Received 26 February 1970

Abstract

Lindahl, P E and J Surowiak *The circadian fluctuations of the amount of free phosphate and of the activity of acid phosphatase in the kidneys of mice and the effect of UV radiation upon this rhythm* Acta physiol scand 1970 80 254—268

The activity of acid phosphatase and the content of P_i[1] were estimated in kidney homogenates from male and female mice The determinations were repeated at 6 hr intervals during the [illegible] s previously subjected to a high dose of [illegible] of [illegible] kidney homogenate varies in an analogous ma[illegible] changes both in the enzyme activity and in the content of inorganic phosphate In irradiated animals the new maxima in the activity of acid phosphatase were generally accompanied by maxima in the amount of P_i It is supposed that the end products of the reactions in the skin formed under the influence of UV radiation and associated with the degradation of phospholipids are the main factors responsible for the observed changes in the kidney

Almost all physiological processes occurring in animal organisms show some rhythmic variability of their intensity, related either to the diurnal sequence day/night or to changes of the seasons of the year Experimental achievements in this field have been described in detail by Sollberger (1965) and Aschoff (1965), while a recent paper by Surowiak (1969) has provided some supplementary data

The activity of acid phosphatase shows certain rhythmic fluctuations depending on the seasons of the year the intensity of light or the length of day and night Quantitative alterations in the activity of this enzyme in the hypothalamus-pituitary system of the sparrow as a consequence of stimulation by light were reported by Kobayashi and Farmer (1960) Similar changes in the pituitary, thyroid and adrenal glands of mice kept for prolonged periods of time in darkness or light, as well as

[1] Abbreviation P_i=inorganic phosphate
* Present adress Departmetn of Animal Physiology Jagiellonian University Kraków, Poland

after irradiation of the animals with a very intense white light, were observed by Surowiak and Tilgner (1968)

Finally, Surowiak (1969) demonstrated marked daily variations in the activity of this enzyme in the hypothalamus and the pituitary, thyroid and adrenal glands of mice and showed that this rhythm is altered by exposure to UV or X radiation

The activity of acid phosphatase shows daily changes in the neurohypophysis the gland releasing the antidiuretic hormone It seems therefore of interest to study whether similar changes in the activity of this phosphatase occur in the kidney, the physiological activity of which is partly controlled by this hormone, and whether these changes are correlated in time with those in the neurohypophysis It was the aim of the present study to investigate by means of chemical analysis the variation in P_i and in the activity of acid phosphatase during the day and the influence of UV radiation upon these variations in the kidney of the mouse

Material and methods

In each experiment 126 inbred albino mice aged 8 to 12 weeks were used The sex ratio of the animals was 1 1 The mean weight of the females was 23 6±1 88 g and that of the males 27 83±2 66 g The animals were reared in a windowless room in artificial light between 08 00 and 20 00 After 10 days of adoption of the animals to this light rhythm the following experiments were carried out.

... vity of acid phosphatase
... ry 6 hrs starting from
... (at 12 00 18 00 24 00

In the experimental group consisting of 35 males and 35 females identical determinations were carried out with animals previously treated with UV radiation between 10 00 and 11 00

... al dislocation and decapitation
... ition containing 0 05 M acetate
... ucrose solution the organ was
... 1955 Karlsson 1969) and the
homogenate was centrifuged at 800 g for 20 min at 4° C Under these conditions the homogenate separates into two fractions the supernatant quite limpid and bright yellow in

two determinations

The amounts of P_i are given as μmol/g wet tissue and the activities of the acid phosphatase as μmol of liberated phosphate/g wet tissue/10 min

In preliminary experiments "activation (Wattiaux and de Duve 1956 Wattiaux *et al* 1956) of the acid phosphatase in the homogenate was shown to occur only in the sediment fraction Activation was attained in these experiments by incubating 2 0 ml of homogenate for 45 min at 37° C with 1 0 ml of a 0 1 % solution of Triton X 100 in 0 05 M acetate buffer pH 5 0 de Duve Wattiaux and Wibo 1962 Sawant *et al* 1964 Karlsson 1969) Hence the activity of acid phosphatase in the supernatant was measured directly without previous incubation with Triton

To sum up the activity of acid phosphatase and the amount of P_i were analyzed in

TABLE I P_i (μmol/g wet tissue) in kidney homogenates from male and female mice C control animals UV, irradiated animals Figures in brackets give numbers of animals in groups. s = standard deviation

Time of sacrifice of animals		Males Mean s	Significance of difference between C and UV	Females Mean s	Significance of difference between C and UV	Significance of difference between control females and males
12.00	C	22.74 ± 1.27 (7)		24.44 ± 1.47 (7)		$p < 0.05$
	UV	20.19 ± 1.79 (7)[1]	$p < 0.01$	22.01 ± 0.97 (7)	$p < 0.005$	
18.00	C	21.73 ± 1.15 (7)		23.87 ± 1.88 (7)		$p < 0.025$
	UV	21.02 ± 1.57 (7)		22.34 ± 1.22 (7)		
24.00	C	22.27 ± 0.58 (7)		23.94 ± 1.96 (7)		$p < 0.05$
	UV	22.33 ± 1.30 (8)[1]		22.81 ± 1.28 (7)		
06.00	C	21.70 ± 0.85 (7)		24.06 ± 1.68 (7)		$p < 0.01$
	UV	21.34 ± 1.10 (7)		23.25 ± 1.34 (7)		
12.00	C	22.74 ± 1.27 (7)		24.44 ± 1.47 (7)		$p < 0.05$
	UV	21.41 ± 1.31 (7)	$p < 0.05$	21.83 ± 1.85 (6)	$p < 0.02$	

[1] Significance of difference between extreme values of the group $p < 0.01$

Results

P_i in the kidney homogenate

The content of P_i in the homogenate of the kidney of the control male mouse exhibited daily fluctuations with maxima at 12.00 and 24.00 and minima at 18.00 and 06.00 (Table I Fig 1) After irradiation this rhythm was changed A very low minimum appeared at 12.00 i.e. 1 hr after exposure and was followed by a second smaller one 18 to 24 hrs later The differences between the values of the control and the experimental groups at 12.00 and 24 hrs later were statistically significant ($p <$ 0.01 and $p < 0.05$ respectively) (Table I Fig 1)

In the control group of females the content of P in the homogenate also shows daily variations reaching a maximum at 12.00 and a minimum between 18.00 and 06.00 The differences between the extreme values are however not statistically significant (Table I Fig 2) The contents of P_i in the homogenates from irradiated females are at all points of time lower than in the corresponding control animals Two minima appeared at 12.00 and 24 hrs later as well as a maximum at 06.00 The differences between the values for the control and experimental groups at 12.00 and 24 hrs later are statistically significant (Table I Fig 2)

On comparing the contents of P_i in the kidney homogenates from untreated males and females it becomes clear that all the mean values for females were lower than the corresponding ones for males and that the differences between these means are statistically significant (Table I Fig 1 and 2)

P_i in the supernatant

The graphical representation of the variations of the contents of P_i in the supernatants from control males was similar to the curve illustrating the corresponding

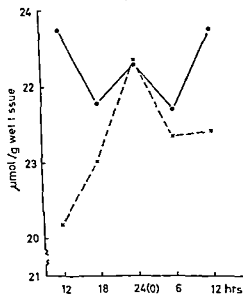


Fig 1

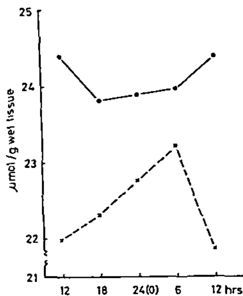


Fig 2

Fig 1 and 2 Amount of P_i (mean value) in unfractionated kidney homogenates of male (Fig 1) and female (Fig 2) mice, as a function of time The black circles represent control animals the crosses irradiated animals

TABLE II P_i (μmol/g wet tissue) in the supernatant fractions of kidney homogenates from male and female mice C, control animals UV, irradiated animals. Figures in brackets give numbers of animals in groups s = standard deviation.

Time of sacrifice of animals		Males Mean s	Males Significance of difference between C and UV	Females Mean s	Females Significance of difference between C and UV	Significance of difference between control females and males
12 00	C	13 36 ± 0 54 (7)[1]		13 63 ± 0 86 (7)		
	UV	11 76 ± 1 21 (7)[2]	$p<0\ 01$	12 13 ± 0 80 (7)	$p<0\ 005$	
18 00	C	13 04 ± 0 50 (7)		13 54 ± 0 97 (7)		
	UV	12 51 ± 0 68 (7)		12 74 ± 1 16 (7)		
24 00	C	13 30 ± 0 62 (7)		12 69 ± 1 48 (7)		
	UV	13 09 ± 0 84 (8)[2]		12 30 ± 0 99 (7)		
06 00	C	12 69 ± 0 61 (7)[1]		13 57 ± 1 39 (7)		
	UV	12 12 ± 0 70 (7)		12 48 ± 0 88 (7)		
12 00	C	13 36 ± 0 54 (7)[1]		13 63 ± 0 86 (7)		
	UV	12 36 ± 0 33 (7)	$p<0\ 002$	11 94 ± 1 22 (6)	p 0 02	

[1] Significance of difference between extreme values of the group $p \approx 0\ 05$
[2] Significance of difference between extreme values of the group, $p < 0\ 05$

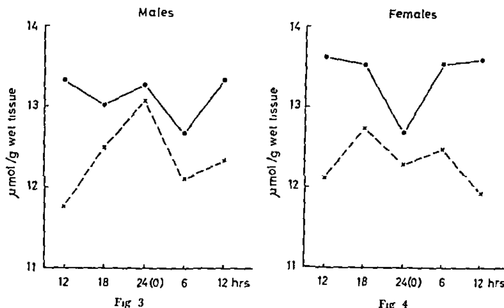


Fig 3

Fig 4

Fig 3 and 4 Amount of P_i (mean value) in the supernatant fractions of kidney homogenates of male (Fig 3) and female (Fig 4) mice, as a function of time Black circles control animals crosses, irradiated animals

values in the unfractionated homogenate, but the figures for the supernatant were lower by almost 50 % than those for the unfractionated homogenate (Table II, Fig 3)

Irradiation brought about a similar displacement of the values for P_i as for the unfractionated homogenate and the differences between the values at 12 00 (1 hr after the exposure) of the experimental and control groups and 24 hrs later were statistically significant The differences at 18 00 and 06 00 were not significant (Table II, Fig 3)

For the control females, the smallest amount of P_i in the supernatant was recorded at 24 00 and the highest at 12 00 but the difference between these values was not statistically significant (Table II Fig 4) For the experimental group of females all the values of the content of P_i in the supernatant were lower than the corresponding values for the control group However statistically significant differences were found only at 12 00 and at 12 00 on the following day i e 7 and 25 hrs after irradiation respectively (Table II Fig 4)

From a comparison between the figures for control males and females it appears that, except at 24 00 when males show a maximum and females a minimum in the content of P_i, all the mean values for males are lower than the corresponding values for females The differences are, however, negligible (Table II Fig 3 and 4)

TABLE III P_i (μmol/g wet tissue) in the sediment fractions of kidney homogenates from male and female mice C, control animals, UV, irradiated animals Figures in brackets give numbers of animals in groups s = standard deviation

Time of sacrifice of animals		Males Mean s	Significance of difference between C and UV	Females Mean s	Significance of difference between C and UV	Significance of difference between control females and males
12 00	C	9 38 ± 0 78 (7)		10 80 ± 0 78 (7)		$p < 0\ 01$
	UV	8 43 ± 0 63 (7)[1]	$p < 0\ 025$	9 88 ± 0 63 (7)	$p < 0\ 05$	
18 00	C	8 69 ± 0 73 (7)		10 32 ± 1 29 (7)		$p < 0\ 02$
	UV	8 51 ± 1 02 (7)		9 74 ± 1 09 (7)		
24 00	C	8 97 ± 0 33 (7)		11 25 ± 0 94 (7)		$p < 0\ 001$
	UV	9 24 ± 0 61 (8)[1]		10 51 ± 1 18 (7)		
06 00	C	9 01 ± 0 60 (7)		10 49 ± 0 83 (7)		$p < 0\ 005$
	UV	9 22 ± 0 60 (7)		10 77 ± 1 02 (7)		
12 00	C	9 38 ± 0 78 (7)		10 80 ± 0 78 (7)		$p < 0\ 01$
	UV	9 05 ± 1 06 (7)		9 97 ± 0 82 (6)	$p < 0\ 05$	

[1] Significance of difference between extreme values of the group, $p < 0\ 05$

P_i in the sediment

The contents of P_i for the control males at every sixth hour of the day are shown in Table III and Fig 5

The minimum exhibited (Fig 1) by the unfractionated homogenate at 06 00 and also shown by the corresponding supernatant (Fig 3) did not appear in the sediment fraction Instead the lowest value was found at 18 00

The effect of UV radiation on the content of P_i in the sediment from the male kidney manifested itself at 12 00 as a significant decrease in relation to the value for

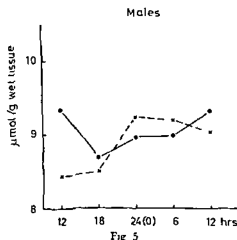


Fig 5

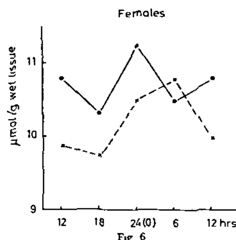


Fig 6

Fig 5 and 6 Amount of P_i (mean value) in the sediment fractions of kidney homo male (Fig 5) and female (Fig 6) mice, as a function of time Black circles con crosses, irradiated animals

TABLE IV Activity of acid phosphatase (μmol/g wet tissue/10 min) in unfractionated kidney homogenates from male and female mice
C, control animals, UV irradiated animals Figures in brackets give numbers of animals in groups s — standard deviation

Time of sacrifice of animals		Males Mean s	Significance of difference between C and UV	Females Mean s	Significance of difference between C and UV	Significance of difference between control females and males
12 00	C	16 22 ± 0 82 (7)		16 03 ± 1 04 (7)		
	UV	16 34 ± 0 71 (7)		15 71 ± 1 11 (7)		
18 00	C	16 96 ± 0 85 (7)[1]		16 89 ± 1 44 (7)[3]		
	UV	17 21 ± 0 97 (7)		16 69 ± 1 36 (7)[4]		
24 00	C	15 85 ± 0 66 (7)[2]		16 33 ± 0 98 (7)		
	UV	18 36 ± 1 62 (8)[2]	$p < 0 005$	15 92 ± 1 94 (7)		
06 00	C	15 93 ± 1 08 (7)		15 46 ± 1 06 (7)[3]		
	UV	15 54 ± 0 91 (7)[1]		16 18 ± 1 46 (7)		
12 00	C	16 22 ± 0 82 (7)		16 03 ± 1 04 (7)		
	UV	17 51 ± 1 11 (7)	$p < 0 05$	14 92 ± 1 38 (6)[4]		

[1] Significance of difference between extreme values of the group, $p < 0 02$
[2] Significance of difference between extreme values of the group, $p < 0 02$
[3] Significance of difference between extreme values of the group, $p \approx 0 05$
[4] Significance of difference between extreme values of the group $p < 0 05$

the control group On the other hand, the maximum appearing between 24 00 and 06 00 only slightly exceeded the values for the control group (Table III Fig 5)

The shape of the curve (Fig 6) illustrating the amounts of P_i in the sediments from control females is reversed, compared with the corresponding curve (Fig 4) for the supernatants The difference between the extreme values of the first mentioned curve is not statistically significant (Table III Fig 6)

Irradiation with UV rays markedly decreased the amount of P_i in the sediment fraction 1 hr, 7 hrs 13 hrs and 25 hrs after exposure shifting the maximum from 24 00 to 06 00 (19 hrs after irradiation) Statistically significant differences between the mean values of this group and the control group were found only at 12 00 and 12 00 the next day (*i e* 1 hr and 25 hrs after irradiation) (Table III, Fig 6)

The data collected in Table III and illustrated by Fig 5 and 6 show that all the mean values of P_i in the sediment fraction from control males were *significantly* lower than the corresponding values for females Also the rhythm of daily fluctuations was different The sediment fraction contained, on an average 41 % of the total amount of P_i present in the unfractionated homogenate

Activity of Acid Phosphatase

Activity of acid phosphatase in unfractionated homogenate

Marked fluctuations during the day of the activity of this enzyme were found for the control group of males, as appears from Table IV and Fig 7 The highest ac-

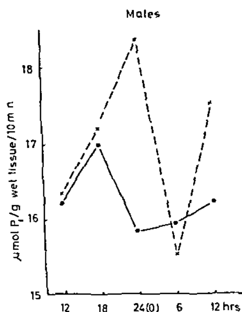


Fig 7

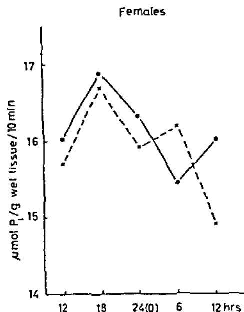


Fig 8

Fig 7 and 8 Activity of acid phosphatase (mean value) in unfractionated kidney homogenates of male (Fig 7) and female (Fig 8) mice as a function of time Black circles control animals crosses irradiated animals

tivity was observed at 18 00 and the lowest at 24 00 The difference between these two values was statistically significant

The effect of UV radiation on the phosphatase activity was pronounced Until 18 00 or during the first 7 hrs after exposure the values of the enzymatic activity for the experimental animals were similar to those for the control group but later at midnight a high peak appeared The difference between this peak and the simultaneous minimum found for the control animals is highly significant ($p < 0\ 005$) The high value observed at midnight decreased later a very low minimum even lower than the control value appearing at 06 00 Finally 24 hrs after exposure a second maximum was observed (Table IV Fig 7)

In the kidney homogenate from control females the highest activity of acid phosphatase was also observed at 18 00 whereas the lowest appeared at 06 00 the significance of the difference between these two values being low (Table IV Fig 8) The effect of UV irradiation on the activity of the enzyme manifested itself in the appearance of a second maximum at 06 00 (19 hrs after exposure) and by a marked decrease of the activity 25 hrs after irradiation (Table IV Fig 8)

Changes in the activity of acid phosphatase in the kidney homogenates from untreated males and females were similar but the minimum appeared 6 hrs later for the females than for the males that is at 06 00 instead of at 24 00 (Table IV Fig 7 and 8)

TABLE V Activity of acid phosphatase (μmol/g wet tissue 10 min) in the supernatant fractions of kidney homogenates from male and female mice
C, control animals UV irradiated animals Figures in brackets give numbers of animals in groups s = standard deviation

Time of sacrifice of animals		Males Mean s	Males Significance of difference between C and UV	Females Mean s	Females Significance of difference between C and UV	Significance of difference between control females and males
12.00	C	6.26 ± 0.81 (7)		5.44 ± 0.96 (7)		
	UV	6.62 ± 0.66 (7)		5.66 ± 0.91 (7)		
18.00	C	6.82 ± 0.78 (7)		6.02 ± 1.70 (7)		
	UV	7.09 ± 0.71 (7)		6.36 ± 1.70 (7)[1]		
24.00	C	6.41 ± 0.48 (7)		5.45 ± 0.73 (7)		$p < 0.02$
	UV	7.69 ± 0.74 (8)[1]	$p < 0.002$	5.23 ± 0.95 (7)		
06.00	C	6.40 ± 0.52 (7)		4.82 ± 0.57 (7)		$p < 0.001$
	UV	6.09 ± 0.66 (7)[2]		5.00 ± 0.43 (7)		
12.00	C	6.26 ± 0.81 (7)		5.44 ± 0.65 (6)		
	UV	7.16 ± 0.63 (7)	$p < 0.05$	4.69 ± 0.65 (6)[2]		

[1] Significance of difference between extreme values of the group $p < 0.001$
[2] Significance of difference between extreme values of the group $p < 0.05$

Activity of acid phosphatase in the supernatant fraction of kidney homogenates

For control males the activity of the enzyme in the supernatant fraction constitutes on an average 38.5 % of the total activity in the homogenate The mean values obtained also show fluctuations in the activity during the course of the day The greatest activity was recorded at 18.00 but later a marked decrease was observed and a low level of enzyme activity persisted until 12.00 the next day (Table V Fig 9)

UV irradiation resulted in identical alterations of the activity of acid phosphatase in the supernatant (Table V Fig 9) as in the unfractionated homogenate (Table IV Fig 7

For the control females on an average 35.0 % of the total activity of acid phosphatase was present in the supernatant fraction The picture of the changes during the day in the enzymatic activity in the supernatant fraction was very similar to that of the unfractionated homogenate The difference between the extreme values of the activity of acid phosphatase was not significant. A certain decrease in the activity was recorded in the supernatant from irradiated mice 24 hrs after the exposure to UV radiation. but this difference was not significant (Table V Fig 10

In general the course of the daily changes in the activity of acid phosphatase in the supernatants from control males and females is similar Measurements of enzymatic activity yielded however consistently higher values for males than for females at corresponding points of time the differences at 24.00 and 06.00 being statistically significant Table V Fig 9 and 10

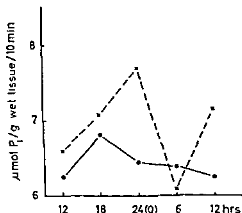


Fig 9

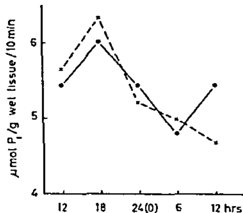


Fig 10

Fig 9 and 10 Activity of acid phosphatase (mean value) in the supernatant fractions of kidney homogenates of male (Fig 9) and female (Fig 10) mice, as a function of time Black circles, control animales crosses, irradiated animals

Activity of acid phosphatase in the sediment fractions of kidney homogenates

This fraction from the control group of males contained, on an average, approximately 61.5 % of the activity of the acid phosphatase in the corresponding homogenate The picture of the daily variations in the activity of the enzyme in the sediment fractions, both from the control and from the experimental animals, was similar to

TABLE [illegible]

Time of sacrifice of animals		Males: Mean ± s	Significance of difference between C and UV	Females: Mean ± s	Significance of difference between C and UV	Significance of difference between control females and males
12.00	C	9.96 ± 0.44 (7)		10.58 ± 0.71 (7)		$p < 0.05$
	UV	9.71 ± 0.73 (7		10.05 ± 0.92 (7)[1]		
18.00	C	10.14 ± 0.95 (7		10.88 ± 0.70 (7)		
	UV	10.13 ± 0.56 (7)		10.32 ± 0.93 (7)		
24.00	C	9.43 ± 0.55 (7)		10.88 ± 0.63 (7)		$p < 0.001$
	UV	10.67 ± 1.37 (8)	$p < 0.05$	10.68 ± 1.42 (7)		
06.00	C	9.60 ± 1.13 (7)		10.64 ± 0.93 7		
	UV	9.54 ± 0.85 (7)		11.86 ± 1.42 7[1]		
12.00	C	9.96 ± 0.44 (7)		10.58 ± 0.71 7		
	UV	10.33 ± 1.10 (7)		10.22 0.82 6		

[1] Significance of difference between extreme values of the group $p < 0.02$

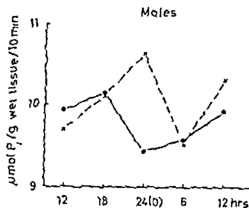


Fig. 11

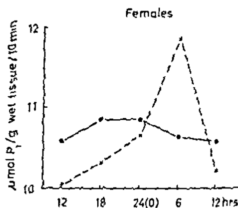


Fig. 12

Fig. 11 and 12. Activity of acid phosphatase (mean value) in the sediment fractions of kidney homogenates of male (Fig. 11) and female (Fig. 12) mice, as a function of time. Black circles control animals, crosses irradiated animals.

that in the corresponding unfractionated homogenates (Tables IV and VI, Fig. 7 and 11).

As much as 65.0 % of the activity of acid phosphatase was associated with the sediment fraction from the control females. Here the fluctuations during the day were partly different from those in the unfractionated homogenate (Table IV and VI, Fig. 8 and 12) in that the greatest activity appeared at 18.00 and persisted until 24.00 whereas the least was recorded at 12.00 (Table VI, Fig. 12).

The effect of UV irradiation on the females was very characteristic. The maximum activity of acid phosphatase found in both the unfractionated homogenate and in the supernatant at 18.00 disappeared completely and a new, very pronounced maximum appeared at 06.00 (Tables IV, V and VI, Fig. 8, 10 and 12). The differences between the extreme enzyme activities in the experimental material were statistically significant (Table VI, Fig. 12).

The values of acid-phosphatase activity in the sediment fractions from males were generally lower than the corresponding values from females, the differences at 12.00 and 24.00 being statistically significant (Table VI, Fig. 11 and 12). Also the graphs of these variations differed for males and females.

Discussion and Conclusions

The results presented above demonstrate marked rhythmic fluctuations of the activity of acid phosphatase in the kidneys of mice of both sexes. The greatest activity of this enzyme was found at 18.00, both for males and females, while the lowest value appeared for males between midnight (24.00) and 06.00, and for females at 06.00.

By means of cytophotometric determinations on histochemical preparations stained with the colour reaction for acid phosphatase according to Gomori (1950) Surowiak (1969) demonstrated circadian rhythmic fluctuations in the activity of this enzyme in the hypothalamus and the pituitary adrenal and thyroid glands of mice both males and females UV and X irradiations disturbed this rhythm to a certain extent

For the males the curve demonstrating the changes during the day in the activity of acid phosphatase in the kidney is very similar to those representing the mean activities of this enzyme in the hypothalamus and the pars nervosa of the pituitary gland with the maxima and minima coinciding

For the group of females on the other hand the curve representing the activity of acid phosphatase in the hypothalamus and neurohypophysis is a reflected image of the curve depicting the mean activity of the enzyme in the kidney homogenates from females These relationships and the fact that the activity of the acid phosphatase in the unfractionated homogenate of the kidneys from males and females are almost identical at the particular times of the day suggest that the diurnal variations in this activity are induced by some exogeneous factor for example the rhythmic changes between dark and light

Extensive studies based on chemical methods (Appelmans and de Duve 1955 Gianetto and de Duve 1955 Appelmans Wattiaux and de Duve 1955 Wattiaux and de Duve 1955 1956 Wattiaux *et al* 1956 Bendall and de Duve 1960 de Duve Wattiaux and Wibo 1962 de Duve 1963 de Duve and Wattiaux 1966) as well as studies based on histochemical methods (Novikoff 1961 de Duve 1963), have proved that acid phosphatase is associated with subcellular structures the lysosomes These organelles fulfil the function of digestive sacs The appearance of acid phosphatase outside the lysosomes in the cytoplasm is looked upon as a pathological phenomenon (also observed by Shamberger and Rudolph 1967) and leads to the death of the cell

In the light of these data an acid phosphatase in the supernatant fraction of the kidney should originate either from the lysosomes phagolysosomes or digestive vacuoles disrupted during homogenization (de Duve 1963 de Duve and Wattiaux 1966) or should perhaps represent enzyme molecules freshly synthesized and not yet enclosed in the lysosomes (storage granules cf de Duve 1963 de Duve and Wattiaux 1966 The synthesis of the enzyme occurs on the ribosomes of the system of the rough endoplasmic reticulum of Novikoff (de Duve 1963) Then the enzyme is transported through the tubules of the smooth endoplasmic reticulum to the region of the Golgi complex where the primary lysosomes are formed (de Duve 1963 de Duve and Wattiaux 1966) A third possibility may be the release of acid phosphatase from the lysosomes in cells surrounding the convoluted tubules in the kidney cortex resulting from the re-absorption by pinocytosis of compounds increasing the permeability of the lysosome membrane It should again be pointed out that supernatant fraction did not show any activation of the acid phosphatase incubation with Triton X 100 Nevertheless it contained a relatively large

tion of the enzyme activity (approximately 38 % in males and 35 % in females) Possibly a part of this fraction of the enzyme activity corresponds to the diffuse 'phosphatase staining of some cell regions in histochemical preparations, which is generally regarded as an artifact Elucidation of the origin of the enzyme in the supernatant will require further detailed studies

UV irradiation produced statistically significant changes in the daily rhythm of the activity of acid phosphatase in the kidney Although the animals of both sexes were affected to some extent, the effect in the males was more pronounced Here no significant alterations of the activity were observed during the first 7 hrs after the exposure Later an increase appeared reaching its maximum 13 hrs after irradiation whereas for the control animals the enzyme activity was at its lowest value at that time of the day, 19 hrs after the exposure the activity suddenly fell below that of the control group and again rose 6 hrs later The graphical picture thus shows a displacement to the right of both the maximum and the minimum with simultaneous elevation and depression respectively of these two values and the appearance of a new maximum 25 hrs after exposure

In females exposed to UV radiation the activity of acid phosphatase was slightly lower than in the controls for the first 12 hrs after the irradiation but the shape of the curve was not altered Only after 19 hrs (at 06 00) did a new, second maximum appear Then the activity decreased again This second maximum which is particularly well developed in the sediment fraction is totally absent in the supernatant fraction which instead exhibits a sharp maximum at 18 00

The induced changes in the activity of the acid phosphatase in the kidneys of animals subjected to a large dose of UV radiation are at present poorly understood However some facts may be stressed Under the influence of large doses of UV radiation the changes in the content of P_i in the homogenate show great similarity to the curves depicting the changes of the activity of acid phosphatase in the kidneys of the same animals Thus in males (Fig 1 and 7) after an initial sudden fall in the content of P UV irradiation promotes its steady rise until midnight when the activity of acid phosphatase also reaches a maximum Later both the amount of P and the enzyme activity markedly decrease A similar simultaneousness was observed in the homogenates from irradiated females Thus at 06 00 (19 hrs after exposure) the greatest mean amount of P_i was observed and this coincided with a second peak of enzymatic activity resulting from irradiation of the animals The same regularity was found also in the studied fractions of the kidney homogenate

Changes in the content of P in the kidney are undoubtedly related to alterations of the carbohydrate and phospholipid metabolism in the organisms of irradiated animals Morton (1953) Turner and Turner (1960) de Duve (1963) and de Duve and Wattiaux (1966 provided evidence that acid phosphatase is involved in this metabolism liberating phosphate from monophosphate esters or transferring it for example to glucose (Morton 1953)

UV irradiation certainly changes to a certain extent the metabolism of the animal organism by photochemical interaction with the skin As a result some products are

formed and are either distributed with the blood or act directly on the free nerve endings of the autonomic nervous system in the skin. The vascular bed becomes dilated by nervous reflexes

Johnson and Mier (1967) Johnson (1968) and Ticner (1963) observed in the UV irradiated skin apart from damage to the lysosomes (Johnson 1968) and leakage of acid phosphatase into the cytoplasm of skin cells or even into the blood a significant decrease in the contents of phospholipids The substances released from the skin should be transported to the liver and the kidney In the kidney some of them may be excreted with the urine or partially re absorbed in the convoluted tubules The cytoplasm of the cells surrounding these tubules contains numerous lysosomes with acid phosphatase (de Duve and Wattiaux 1966) The lysosomes may fuse with vacuoles formed in the process of pinocytosis during re absorption yielding digestive vacuoles These structures are known to be involved in the process of intracellular degradation of various substances possibly also including products that are formed by photochemical processes in the irradiated skin and are effective as substrates of the acid phosphatase Accumulation of a substance effective as a substrate may stimulate increased synthesis of an enzyme by induction (*cf* West *et al* 1966 p 468) The observed sudden rises in the activity of acid phosphatase and in the amount of P_i in the kidney homogenates of irradiated animals 13 hrs after exposure are thus comprehensible

An increased supply of phospholipid derivatives may also be due to another reaction It is known that UV irradiation may penetrate the skin to a depth of 0 5 mm (Blum 1959 Surowiak 1965) Hence one should consider also the possible effect of irradiation on phospholipids in the blood circulating through the skin capillaries of the ears and the tail The amount of blood lipids accessible to irradiation is increased by dilatation of these capillaries during the period of irradiation a reaction brought about by histamine and histamine like substances formed in the skin in the photochemical reaction splitting off CO from histidine (Pfenningsdorf and Schreiber 1960)

Finally UV irradiation transforms 7 dehydrocholesterol into vitamin D_3 which promotes the deposition of P_i in the bones Thus the consumption in the body of P should be expected to increase This may contribute to the fall in the content of P in the kidney homogenate during the first few hours after exposure

We are very much indebted to Dr H O Karlson for introducing one of us (J S to the analytical methods used

References

APPELMANS F and C DE DUVE Tissue fractionation studies 3 Further observations on the binding of acid phosphatase by rat liver particles *Biochem J* 1955 59 426—433

APPELMANS F R WATTIAUX and C DE DUVE Tissue fractionation studies 5 The association of acid phosphatase with a special class of cytoplasmic granules in rat liver *Biochem J* 1955 59 438—445

ASCHOFF J *Circadian clocks* North Holland Publ Co Amsterdam 1965 pp 479

Bendall, D S and C de Duve Tissue fractionation studies 14 The activation of latent dehydrogenases in mitochondria from rat liver *Biochem J* 1960 *74* 444—450

Blum, H F, *Carcinogenesis by ultraviolet light* Princeton University Press, Princeton New Jersey 1959 pp 340

Duve, C de, The lysosome *Scient Amer* 1963 *208* 64—72

Duve C de and R Wattiaux, Functions of lysosomes *Ann Rev Physiol* 1966 *28* 435—492

Duve C de R. Wattiaux and M Wibo *Effect of fat soluble compounds on lysosomes in vitro Biochem Pharmacol* 1962 *9* 97—116

Gianetto R. and C de Duve Tissue fractionation studies 4 Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat liver particles *Biochem J* 1955 *59* 433—438

Johnson B E and P Mier Phospholipids and the reaction of skin to ultra violet radiation *Nature* (Lond) 1962 *194* 101—102

Johnson B E Ultraviolet radiation and lysosomes in skin *Nature* (Lond) 1968 *219* 1258—1259

Karlsson H-O In vitro studies of the stability of liver lysosomes after in vivo treatment of rats with sulfapyridine *Experientia* (Basel) 1969 *25* 1290—1291

Kobayashi H and D S Farner The effect of photoperiodic stimulation on phosphatase [illegible] hite-crowned sparrow Zonotrichia [illegible]

M[illegible] *Nature* (Lond) 1953 *172* 65—68

Nc[illegible] *cell* (Ed J Brachet and A. E-[illegible] 423—488

Pfenningsdorf G and H Schreiber Zur Kenntnis des Histidin Histamin Prozesses *Probl Ergeb Biophys Strahlenbiol* II Bericht 13—15 Oktober 1958 Akad Verl Berlin 1960 170—173

Sawant P L S Shibko U S Kumta and A L Tappel Isolation of rat liver lysosomes and their general properties *Biochim biophys Acta* Amst 1964 *85* 82—92

Shamberger R J and G Rudolph Increase of lysosomal enzymes in skin cancers *Nature* Lond 1967 *213* 617—618

Sollberger A. *Biological rhythm research* Elsevier Publ Co Amsterdam, London and New York 1965 pp 461

Surowiak J Influence of UV radiation on the postembryonal development of the testes of the white mouse *Mus musculus L Vest Ceskoslov Spol Zool Acta Soc Zool Bohemoslov* 1965 *29* 255—281

Surowiak J Quantitative changes of acid phosphatase in the hypothalamus pituitary adrenals and thyroid of mice (*Mus musculus L* exposed to large single doses of UV er V rays taking into account the circadian rhythm *Folia Biol* Warz 1969 *17* 105—140

Surowiak J and S Tilgner. Changes in the quantity of acid and alkaline phosphatase in the pituitary thyroid and adrenals of white mice *Mus musculus L*) exposed to different influences of white light *Acta Biol Crac Zool* 1967 *10* 149—168

Tickner A Changes in the phospholipids of mouse skin after ultraviolet irradiation in vitro *Biochem J* 1963 *88* 80—84

Turner, D H and J F Turner. The hydrolysis of glucose monophosphatase by a phosphatase preparation from pea seeds *Biochem J* 1960 *74* 486—491

Wattiaux R and C de Duve Tissue fractionation studies 7 Release of bound hydrolases by means of Triton X 100 *Biochem J* 1956 *63* 606—608

Wattiaux R P Baudhuin A M Berleur and C de Duve Tissue fractionation studies 8 Cellular localization of bound enzymes *Biochem J* 1956 *63* 608—612

Weissmann G and L Thomas Studies on lysosomes II The effect of cortisone on the release of acid hydrolases from a large granule fraction of rabbit liver induced by an excess of Vitamin A *J clin Invest* 1963 *42* 661—669

West E S W R Todd H S Mason and J T van Bruggen *Textbook of Biochemistry* 4th ed Macmillan Company New York 1966 pp 1595

Acta physiol. scand. 1970 80 269—275

From the Department of Physiology Karolinska Institutet, Stockholm Sweden

Antagonism by Calcium of the Inhibitory Action of Prostaglandin E_2 on Sympathetic Neurotransmission in the Cat Spleen

By

P Hedqvist

Received 26 February 1970

Abstract

Hedqvist, P *Antagonism by calcium of the inhibitory action of prostaglandin E on sympathetic neurotransmission in the cat spleen* Acta physiol scand 1970 *80* 269—275

Experiments were carried out with the isolated perfused cat spleen and bovine splenic nerve [illegible] E_2 did not affect the noradrenaline releasing effect of tyramine Atropine did not alter the inhibitory effect of prostaglandin E_2 on noradrenaline release Prostaglandin E_2 did not change the amplitude of action potentials in bovine splenic nerves There is thus no support for a blocking action on nerve conduction It is concluded that prostaglandin E_2 may prevent calcium from gaining access to those sites in sympathetic nerve terminals where it promotes secretion of noradrenaline

Prostaglandins of the E series (PGE) are known to interfere with the effector response to nerve stimulation in several sympathetically innervated tissues (Berti and Usardi 1964 Brundin 1968 Hedqvist 1968 Euler and Hedqvist 1969) Recent reports indicate that PGE_2 may interfere with the function of the sympathetic neuro-effector system both by inhibition of the effector response to noradrenaline (NA) released and by inhibition of the release of NA from the nerves (Hedqvist and Brundin 1969 Hedqvist 1969 1970 Hedqvist Stjarne and Wennmalm 1970)

So far little is known about the target for PGE_2-induced inhibition of neurotransmitter release from sympathetic nerves (*cf* Hedqvist 1970) The present work was designed to study this issue Experiments were carried out on the isolated perfused cat spleen to study the calcium dependence of the inhibitory effect of PGE_2 on outflow of NA to nerve stimulation, and also to determine the effect of PGE_2 on the release of NA caused by injection of tyramine TA) In view of the annulment by atropine of the inhibitory action of PGE_1 on sympathetic neurotransmission in the guinea pig vas deferens (Euler an

PGE_2 induced inhibition of NA release in response to nerve stimulation was also studied The bovine splenic nerve trunk was used to investigate the effect of PGE_2 on conduction of nerve impulses

Methods

Cat spleen 11 cats of both sexes, weighing 2—4 kg were used for the study The experimental animals were anesthetized with sodium pentobarbital (30 mg/kg i p) and heparinized (1000 I U /kg i v) The abdomen was opened by a midline incision and the splenic vessels and nerves were carefully dissected free as far proximally as possible, and were then cut The spleen was [illegible]

[illegible]

and aerated with 5 per cent CO_2 in O_2 In experiments when the spleen was perfused with [illegible]

[illegible]

into 10 ml portions by means of an automatic fraction collector The splenic nerves were electrically stimulated by platinum electrodes Trains of 200 supramaximal stimuli (2 5 msec 10—15 V 10 pulses/sec) were applied by a Grass S4 stimulator PGE_2 was infused i a to produce a final concentration of 3×10^{-7} M TA was administered i a as rapid infusion (10 μg in 20 sec)

In most experiments the NA stores of the spleen were labelled by i a infusion of 30 μC of ^{3}H dl NA (spec act about 5 C/mmole N E N) to facilitate monitoring of the outflow of total NA from the organ During periods of nerve activity or infusion of TA most of the radioactivity in the effluent from the spleen is known to represent intact NA (Hedqvist Oliverio and Stjarne 1968)

The NA content of representative samples of the perfusate was measured fluorimetrically after purification on alumina (Euler and Lishajko 1961) The radioactivity of the different perfusate fractions was determined by counting 0 5 ml aliquots in a 7 3 toluene absolute ethanol solution containing 4 g of 2 5 diphenyloxazole and 100 mg of 1-4 bis 2(4 methyl 5-phenyloxazolyl)benzene per liter of toluene in a Packard Tri Carb Liquid Scintillation Spectrometer Quenching was monitored by internal standards of ^{3}H dl NA

Splenic nerves Bovine splenic nerves were obtained at the slaughter house within 30 min post mortem and immediately chilled with ice The nerves were carefully dissected free from contaminating tissue and were in most cases desheathed They were then placed in a nerve chamber with the midportion of the nerves kept in Krebs Henseleit s of the above mentioned composition (dextran excluded) The temperature of the chamber was kept constant at 37° C Supramaximal stimulation of the nerves was carried out with platinum electrodes and a Grass S4 stimulator operated at a duration of 2—3 msec and 10—30 V Single pulses or short trains of rectangular pulses (2—3 sec) at a frequency of 10/sec were delivered at 1 min intervals The recording electrodes (platinum) were connected via a cathode follower to a DC preamplifier (Grass P6 12A frequency response 0—10 000 Hz) and a cathode ray oscilloscope (Tektronix 502)

Results

Addition of PGE_2 (3×10^{-7} M) to the medium perfusing the isolated cat spleen was found to markedly depress the outflow of radioactive and endogenous NA in response to nerve stimulation as reported elsewhere (Hedqvist 1969 1970)

Raising the calcium concentration from 2 5 to 5 mM in the medium perfusing the spleen resulted in an increased outflow of NA in response to nerve stimulation in agreement with previous observations in the rabbit heart and the cat spleen (Hukovic and Muscholl 1962 Kirpekar and Misu 1967) The same increase of the concentration of calcium in the perfusion medium markedly counteracted the inhibitory

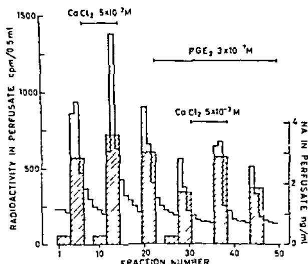


Fig 1 Perfused cat spleen Out flow of radioactivity, drawn line, and of fluorimetrically determined NA, hatched bars, from the spleen, resting and in response to nerve stimulation (200 pulses at 10/sec) Effect of PGE_2 (3×10^{-7} M) and of increasing the calcium concentration in the perfusion medium from 2.5 to 5 mM

action of PGE_2 and restored the outflow of NA to nerve stimulation to normal values (Fig 1)

Intraarterial infusion of TA (10 μg in 20 sec) caused a moderate increase in efflux of both radioactive and fluorimetrically determined NA from the spleen (*cf* Hedqvist, Oliverio and Stjärne 1968) Infusion of PGE_2 (3×10^{-7} M) starting 4—5 min before the *infusion of TA did not materially change this NA efflux while it produced* the expected decrease of NA outflow in response to nerve stimulation (Fig 2)

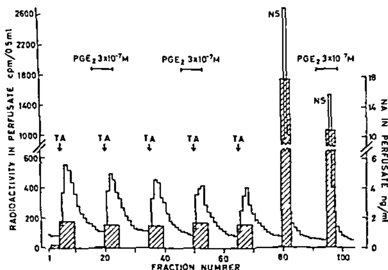


*Fig 2 Perfused cat spleen Outflow of radioactivity, drawn line and of fluorimetrically deter*mined NA hatched bars from the spleen, resting and in response to TA infusion (10 μg 20 sec) or nerve stimulation (200 pulses at 1

In the present study increasing the calcium concentration in the perfusion medium was found to increase the outflow of NA to nerve stimulation thus confirming previous observations in heart and spleen (Hukovic and Muscholl 1962, Kirpekar and Misu 1967) Increasing the calcium concentration was also found to antagonize the inhibitory action of PGE_2 on outflow of NA to nerve stimulation On the other hand the NA releasing effect of TA was not affected by PGE_2 This may be significant since there is overwhelming evidence that transmitter release from sympathetic nerves following excitation is calcium dependent (Hukovic and Muscholl 1962 Burn and Gibbons 1964, Boullin 1967 Kirpekar and Misu 1967) while the release of NA induced by TA apparently is not calcium dependent (Lindmar Loffelholz and Muscholl 1967, Thoenen, Hurlimann and Haefely 1969)

According to the classical theory for excitation secretion coupling in sympathetic nerves depolarization causes an increased influx of calcium which in its turn promotes the release of transmitter (*cf* Ferry 1966) However, an alternative explanation has been offered by Burn and Rand (1959 1965) These authors suggest that acetylcholine is released by the nerve impulse and in its turn increases the permeability of the axonal membrane to calcium The finding that the inhibitory action of PGE_1 on neuromuscular transmission in the guinea pig vas deferens is annulled by atropine is compatible with this concept (Euler and Hedqvist 1969) On the other hand it has not been possible to demonstrate a similar effect of atropine on the inhibitory action of PGE_2 on NA outflow to nerve stimulation in the cat spleen (present results) or in the rabbit heart (Hedqvist Stjarne and Wennmalm 1970) In fact several investigators using the cat spleen preparation have failed to produce any evidence for a cholinergic link in sympathetic neuotransmission (Blakeley Brown and Ferry 1963 Thoenen *et al* 1966 Blakeley and Brown 1968)

In conclusion the present study demonstrates a definite antagonism between calcium and PGE on the outflow of NA to nerve stimulation It is thus tempting to assume that this is the main level at which PGE_2 acts to block release of NA from the sympathetic nerves The action of PGE_2 could be exerted at the level of the axonal membrane by inhibition of calcium influx induced by depolarization. An alternative explanation is that PGE due to its lipid solubility may pass through the cell membrane and prevent calcium from reaching the reactive sites where it is able to promote transmitter relase

This study was supported by grants from the Swedish Medical Research Council project no K70 14X 3027 01A and from Svenska Sallskapet for Medicinsk Forskning Prostaglandin E₂ was kindly given by Dr J Pike Upjohn Co Kalamazoo USA

References

Ar [illegible] and Mg^{++} effects on toad bladder response [illegible] Physiol 1967 213 803—808

Ar [illegible] (PGE_1) escamb del calcio a [illegible] 43 521—524

Berti F and M M [illegible] of free fatty acid mobilization on G Arteriosci 1964 2 261 265

Blakeley A G H and G L Brown Effects of an anticholinesterase on the transmitter over

flow and the response of the spleen to nerve stimulation *J Physiol* (Lond) 1968 *191* 71P—72P

Bl … on the re

Bc … hetic trans

Brundin J The effect of prostaglandin E_1 on the response of the rabbit oviduct to hypogastric nerve stimulation *Acta physiol scand* 1968 *73* 54—57

Br … onse to … 8

Br … 1959 104 105—105

Burn J H and M J Rand Acetylcholine in adrenergic transmission *Ann Rev Pharmacol* 1965 *5* 163—182

Coceani F C Pace Asciak and L S Wolfe, Studies of the effect of nerve stimulation on prostaglandin formation and release in the rat stomach. In *Prostaglandin Symp of the Worcester Foundation for Experimental Biology* Oct 1967 Ed P W Ramwell and J E Shaw Wiley New York 1968 39—45

Coceani F and L S Wolfe On the action of prostaglandin E_1 and prostaglandins from brain on the isolated rat stomach *Canad J Physiol Pharmacol* 1966 *44* 933—950

Emmons P R J R Hampton M J G Harrison A J Honour and J R A Mitchell, Effect of prostaglandin E_1 on platelet behaviour in vitro and in vivo *Brit Med J* 2 468—472

Euler U S v and P Hedqvist Inhibitory action of prostaglandins E_1 and E_2 on the neuromuscular transmission in the guinea pig vas deferens *Acta physiol scand* 1969 *77* 510—512

Euler U S v and F Lishajko Improved technique for the fluorimetric estimation of catecholamines *Acta physiol scand* 1961 *51* 348—355

Fassina G and A R Contessa Digitoxin and prostaglandin E_1 as inhibitors of catecholamine-stimulated lipolysis and their interaction with Ca^{2+} in the process *Biochem Pharmacol* 1967 *16* 1447—1453

Ferry C B Cholinergic link hypothesis in adrenergic neuroeffector transmission *Physiol Rev* 1966 *46* 420—456

Hedqvist P Reduced effector response to nerve stimulation in the cat spleen after administration of prostaglandin E_1 *Acta physiol scand* 1968 *74* 7A

Hedqvist P Modulating effect of prostaglandin E_2 on noradrenaline release from the isolated of cat spleen *Acta physiol scand* 1969 *75* 511—512

Hedqvist P Control by prostaglandin E_2 of sympathetic neurotransmission in the spleen. *Life Sci* 1970 *9* 269—278

Hedqvist P and J Brundin Inhibition by prostaglandin E_1 of noradrenaline release and effector response to nerve stimulation in the cat spleen *Life Sci* 1969 *8* 389—395

Hedqvist P A Oliverio and L Stjärne Inhibition by phenoxybenzamine of the nor

Kayaalp S O and R J McIsaac Absence of effects of prostaglandins E_1 and E_2 on ganglionic transmission *Europ J Pharmacol* 1968 *4* 283—288

Kirpekar S M and Y Misu Release of noradrenaline by splenic nerve stimulation and its dependence on calcium *J Physiol* (Lond) 1967 *188* 219—234

Klaus W and F Piccinini Über die Wirkung … isolierter Meerschweinchenherzen *Experientia*

Kopin I J G R Breese K Krauss and V K … norepinephrine from the cat spleen during symp … *Ther* 1968 *161* 271—278

Mantegazza P La prostaglandina E_1 come sostanza sensibilizzatrice per il calcio a livello … 1965 *20* 66—72

St … lins E_1 E_2 A_1 and F_1 on isolated … 725—733

Th … Haefely Untersuchungen zur Frage eines cholinergischen Gliedes in der postganglionären sympathischen Transmission *Helv physiol pharmacol Acta* 1966 *24* 229—246

Acta physiol. scand. 1970. 80. 276—288

From the Department of Histology, Karolinska Institutet, Stockholm

Ontogenesis of Peripheral Adrenergic Neurons in the Rat: Pre- and Postnatal Observations

By

J DE CHAMPLAIN[1], T MALMFORS, L OLSON and CH SACHS

Received 19 March 1970

Abstract

CHAMPLAIN, J DE, T MALMFORS, L OLSON and CH SACHS *Ontogenesis of peripheral adrenergic neurons in the rat pre- and postnatal observations* Acta physiol scand 1970 *80* 276—288

The early development of the peripheral adrenergic neurons of the rat was followed with the Falck-Hillarp fluorescence technique In the 13 [illegible] ...scence [illegible] ...the next [illegible] well developed adrenergic innervation of the plexus of Auerbach at birth while the [illegible] organs investigated demonstrated well developed networks of adrenergic nerves from about the fourth day on It was concluded from some pharmacological experiments that, under optimal conditions the fluorescence technique can reveal all or almost all adrenergic nerves also during early development

Knowledge of the ontogenesis of the sympathetic nervous system is of fundamental importance for the interpretation of studies of the development of adrenergic neurotransmission

The sympathetic adrenergic nerves, owing to their content of noradrenaline (NA), can be visualized with the histochemical fluorescence method of Falck and Hillarp. Assuming that enough NA is present also during development, this method would be useful in studying the outgrowth of adrenergic nerves To date, only a few reports on this subject have been published Knowledge is lacking above all as to the prenatal appearance of fluorescence in the sympathetic nervous system of mammals

It is the purpose of the present report to describe some aspects of the pre- and

[1] Dr Jacques de Champlain is a Markle Scholar in Academic Medicine address Dept of Physiology, Faculty of Medicine, Univ of Montréal, Montreal 3 P Q, Canada

postnatal development of the sympathetic adrenergic nerves in the rat The problem whether at an early stage, sympathetic nerves exist which cannot be visualized with the fluorescence technique will be elucidated The appearance of fluorescent nerve cells and fibres in relation to the development of the effector organs and the fluorescence morphology of the immature neurons will be described

Material and Methods

Albino rats (Sprague Dawley) of both sexes were used Groups of 4—5 females were placed [illegible]

[illegible]

Controls were run in amine free medium [illegible]

Results

Prenatal observations

Specific green to yellow green catecholamine fluorescence was observed in the embryo towards the end of the second week of pregnancy In a few observations on the 10th—12th day of pregnancy no specific fluorescence could be observed with certainty

A row of loosely arranged cells with generally low but varying, fluorescence intensities was found bilaterally along the dorsal aspect of the embryo toward the end of the second week of pregnancy It consisted of small cells with fluorescent cytoplasm and short fluorescent irregular processes, forming a loose meshwork (Fig 1a) Two groups of more densely fluorescent cells were found arranged in the thoracic and lumbar regions Individual cell processes were not readily observable in these elongated cell masses No fluorescent axon bundles could be traced at this earl

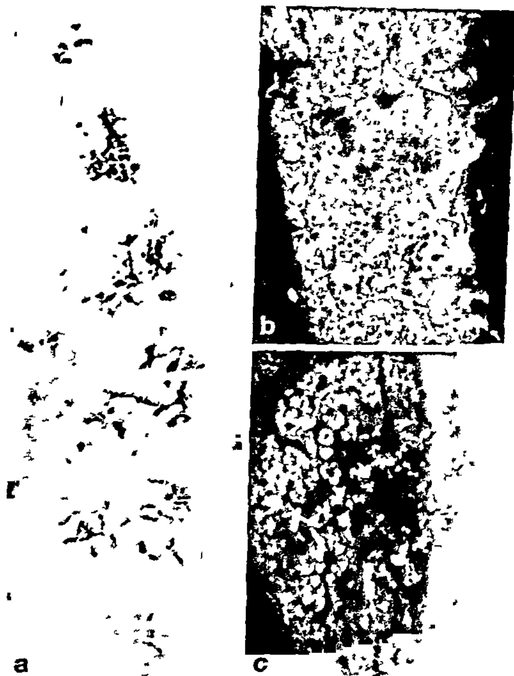


Fig. 1 Monoamine-[illegible] cells in the 13 day rat embryo as revealed by fluorescence microscopy of freeze-dried tissue[illegible]. Primordia of the sympathetic trunk ganglia. Groups of small fluorescent sympathicoblasts with short fluorescent processes are seen along the curvature of the spinal column. Sagittal sec[illegible] whole embryo [illegible] 1 0 b Stellate ganglion. Densely packed small cells with varying flu[illegible]orescence intensities ×300 c Adrenal medulla. Small intensely fluorescent and loosely arra[illegible] medullary cells are found surrounded by the adrenal cortex. ×120

stage, and in fact most organs were identifiable only as small anlage The column of fluorescent cells then showed segmental enlargements These enlargements consisted of groups of cells, and each enlargement showed a slender extension of cells and fibres in the nerves towards the spinal cord

A rapid growth and differentiation of the fluorescent cell system paralleled the rapid increase in body weight which took place during the first half of the third week of pregnancy The paravertebral sympathetic ganglia were clearly visible, and fluorescent axons were still seen extending towards the spinal cord Three large elongated groups of fluorescent cells were observed probably representing the superior cervical ganglion the stellate ganglion (Fig 1 b) and the lumbar plexuses The ganglia consisted of densely packed small cells of varying but moderate fluorescent intensities connected by partly fluorescent fibre bundles Small intensely green to yellow fluorescent cells were scattered in the ganglia

Close to the superior cervical ganglion at the bifurcation of the carotid artery fluorescent cells were observed in the glomus caroticum

Intensely fluorescent medullary cell groups were found in the anlage of the adrenals The majority of these cells had strong fluorescence intensities and a more yellow color while some of the cells had a weaker and greener fluorescence The medullary cells however were not as densely packed as in the adult adrenal medulla (Fig 1 c) Outside the adrenal glands a rounded compact mass of green to yellow green fluorescent cells similar to the paravertebral ganglia was found at the proximo-vertebral margin of the gland in the posterior attachment of the diaphragm A large loosely arranged plexus of cells and fluorescent fibre bundles extended medially over the aorta at the level of the kidneys

Fluorescent axon bundles were found on the 18th day in several larger nerve trunks such as the intercostal nerves in the mediastinum and along the abdominal aorta However, no fluorescent nerve fibres were identified with certainty in effector organs such as the heart lungs liver kidneys intestine pancreas salivary glands or irides

In order to increase the fluorescence intensities and possibly detect fibres which could not be found in the untreated animal a few pregnant rats were treated with the monoamine oxidase inhibitor nialamide (500 mg/kg 4 hrs before killing on the 18th day of pregnancy

Although an increase was noted in the fluorescence intensities of the catechol amine containing structures no or almost no increase in the number of detectable axons occurred in the peripheral tissues

Non neuronal specific fluorescence was observed in the pigment epithelium of the eye and in the first few enterochromaffin cells of the intestine which appeared in the 18 day embryos

During the second half of the last week of pregnancy fluorescent nerve fibres grew out to a varying extent in different organs Axon bundles could be found in the hilus regions of several parenchymatous organs and in the mesentery of the intestine

It is noteworthy that the outgrowing axons looked thicker, smoother and were often of a higher fluorescence intensity than the non terminal axons found in adult control animals

Postnatal observations

Although certain differences were observed between the various organs investigated (iris auricles small intestine salivary glands and vas deferens) the following general statements can be made concerning the fluorescence morphology of the sympathetic nerves during the first postnatal week The outgrowing axons had a stronger fluorescence intensity than nonterminal axons in adult animals and seemed thicker and smoother (*cf* Fig 2 c) They were not completely smooth however but showed elongated irregularly spaced enlargements of varying sizes Growth cones were infrequent

The anlage of the organs existed before any fluorescent nerves were observed within them The nerves were seen to grow from the periphery or from the hilus regions and inward into the developing organs When nerve plexuses were being formed in the effector organs they had initially a very weak fluorescence intensity which gradually increased A typical varicose ground plexus was not formed durin the first week although the incipient formation of nerve terminals with more strongly fluorescent and regularly spaced enlargements was observed after a few days

The development of the sympathetic ganglia was not followed closely after birth The varying but generally low fluorescence intensities increased during the first week and the variability became less pronounced The size of the cells increased

Mast cells were a prominent feature in young organs especially iris and heart

The sympathetic nerves differ from the adult pattern also during the second and third postnatal weeks An intense fluorescence of non terminal axons for instance was still noted in the salivary glands and irides of the 12 day animal There was a gradual decrease in the fluorescence intensity of non terminal axons and a gradual increase in the fluorescence intensity of the nerve terminals where varicosities became more prominent

If one considers separately the various organs investigated certain differences and specific observations are worth mentioning

Iris A very faint plexus of thin nerves mainly located in the sphincter region was noted in the poorly developed organ at birth Mast cells were common. In the 4 day animal the iris was larger and easier to stretch and the adrenergic ground plexus quite well devel ped Fig 2 a Strongly fluorescent axons were found to run circularly in the corpus ciliare and sphincter regions interconnected by radially running axons in the dilat r area A network of thin fibres with faintly outlined varicosities was found over the entire preparation Mast cells were evenly distributed in the three regions Towards the end of the second week non terminal axons were still of higher fluorescence intensity and terminal fibres of lower than in adult (44 day old) animals Mast cells were still seen also over the dilator area By the end of the third week the only difference from the adult pattern was that the meshes of

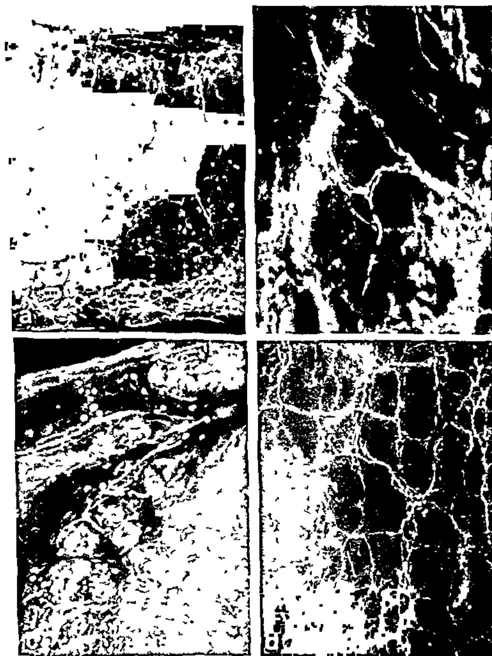


Fig 2 Early postnatal appearan e o he lrene n he cence microscopy a S et l prepar incubated in a metl l NA The whole the sphincter margin (bottom Th e

the ground plexus over the dilator area more often contained single fibres

Auricles of the heart At birth, these small organs were quite difficult to stretch In successful preparations some single fluorescent axons were occasionally observed They were smooth and of moderate fluorescence intensity Mast cells were common 2 days after birth, plexus like ramifications were noted in the periphery of the preparation (Fig 2 b) At four days this plexus was more pronounced although still mainly confined to the periphery of the organ Non terminal axons were strongly fluorescent The characteristic innervation of the blood vessels began to form At 12 days a well developed plexus of nerves was found over the whole tissue The axons were still strongly fluorescent while the varicosities of the forming terminal fibres were less pronounced than in the adult animal By the end of the third week the distribution and pattern of innervation were similar to the adult pattern

Submaxillary sublingual gland Axons of increased fluorescence intensity were observed at birth especially in the hilus region (Fig 2 c) These axons were prominent after two days At four days the parenchyma of the submaxillary gland was penetrated by a meshwork of smooth weakly fluorescent nerves while the sublingual gland remained almost completely devoid of nerve fibres Non terminal axons were strongly fluorescent until the end of the second week An almost adult appearance was found by the end of the third week Although the number of fluorescent fibres in each strand of the ground plexus was at this time increased the density of the plexus was rather decreased owing to the enlargement of the acini and ducts

Vas deferens At birth strongly fluorescent axons were observed in nerve trunks in the surrounding connective tissue The circular smooth muscle layer was barely observable and occasionally a single weakly fluorescent fibre could be observed in this layer Fluorescent nerve cell bodies were noted at the beginning of the duct After two days approximately the outer half of the circular muscle layer was invaded by fluorescent fibres The first signs of varicosities could be seen Toward the end of the first week the entire circular layer was penetrated by fluorescent nerves

Small intestine The gut seemed to develop its adrenergic innervation apparatus earlier than the above mentioned organs Thus even at birth the stretch preparations of the muscle layer revealed a well developed meshwork of fluorescent nerves surrounding the non fluorescent nerve cell bodies of the myenteric plexus of Auerbach Most fibres were smooth and of weak to moderate fluorescence intensities Beaded fibres however were also noted (Fig 2 d Strongly fluorescent axons were located in the mesenterial root of the preparations Also in the submucosal plexus of Meissner which is present in the stretch preparations of the mucosa fluorescent fibres were found to surround non fluorescent nerve cell bodies The density of the fluorescent nerves looked higher in the stretch preparations of the muscle layer than in the mucosa From the fourth day on, fluorescent fibres were also found between the regularly spaced meshes of Auerbach s plexus Fibres were thus found running mainly parallel to the circular muscle layer in an amount which increased with age up to the end of the second week On the other hand the density of the plexus of Auer

bach seemed to decrease with age owing to the relatively larger growth of the non neuronal components of the intestine The yellow fluorescent enterochromaffin cells first observed already several days before birth were now found in increasing amounts During the first postnatal week they were relatively few but by the end of the second week they had become numerous

Effects of reserpine, nialamide and NA incubations

Reserpine caused a complete disappearance of the neuronal fluorescence within 4 hrs in the 4 day old animals

The incubation of different organs from 4 day old animals in Ringer buffer alone caused a slight accentuation of the varicose appearance The addition of NA to the incubation medium especially in the high concentration caused an increased fluorescence intensity in all nerves in all the organs studied (iris auricles small intestine submaxillary glands), and the varicose appearance became even more pronounced The varicosities however were still not as marked as in the adult untreated animals Pretreatment with nialamide abolished most of the varicose appearance of the nerves after NA incubation

When good stretch preparations were obtained and the paraformaldehyde reaction was optimal no certain increase in the number of fluorescent nerve fibres was noted after NA incubation In the stretch preparations from the youngest animals examined however, the nerves were often difficult to visualize in the untreated animals especially the few axons in the auricles from newborn animals NA incubation made it possible constantly to visualize the expected amount of nerves in such cases and it is possible that some few nerve fibres at the early stages could be detected only in this way

Discussion

Technical comments

The specificity and high sensitivity of the fluorescence technique used is well documented (see Corrodi and Jonsson 1967 Thus there is no doubt that the fluorescence observed is due to catecholamines The specificity of the neuronal fluorescence is further supported by the fact that there was no fluorescence after pretreatment with reserpine or 6-hydroxydopamine Sachs *et al* 1970) In adult animals all adrenergic nerves are displayed in their whole length owing to the presence of adequate amounts of NA During the ontogenesis however it is possible that NA concentrations are lower and that the conditions for an optimal fluorescence reaction might be changed It is known that embryological and neonatal material is more difficult to process for fluorescence histochemistry (see *e g* Olson 1967 Bjorklund *et al* 1968 The higher water content may make freeze drying more difficult and the tissues may become more vulnerable after freeze drying The number of transmitter storing dense core vesicles as visualized after permanganate fixation (Richardson 1966) is lower at early developmental stages see *e g* Yamauchi and

Burnstock, 1969), suggesting a lesser amount of transmitter, and less stable intraneuronal stores. It is possible that extragranularly located NA is more easily dislocated by diffusion at various stages of the histochemical process. Also the formed fluorophor diffuses easily. The absence of fluorescent nerves in an embryo or newborn animal must therefore be interpreted with extra care. If newly formed nerves were present which could metabolize or take up NA, it should be possible to increase the amount of visible nerves and detect fluorescent nerves at earlier stages after the administration of NA or inhibition of the metabolism. This has been suggested by Read and Burnstock (1969). Since, however, the pharmacological treatments did not essentially increase the amount of visible nerves and fluorescent fibres were detected at very early stages of the ontogenesis, it seems probable that under the present conditions also the negative results can be trusted. It is thus likely that under optimal conditions all adrenergic nerves are detected in the untreated animal (cf. Olson, 1969).

Prenatal observations

The weight development of the rat embryos examined closely followed the growth curves given by Schumann (1969). This author demonstrated that the chief growth period of the rat, in contrast to that of the mouse, falls within the third week of the gestation period. This fact may explain also the rapid development of the sympathetic nervous system observed during the first half of the third week.

Several other aspects than the general weight gain must be considered, however. Different organs are needed at various times, the intestine for instance earlier than the eyes of the blind born rat; this is in agreement with the present finding of an early intestinal innervation. Similarly, the ductus arteriosus of the human fetus was found to have a well developed varicose plexus of adrenergic nerves at a stage when several other tissues had a more embryonal type of innervation (Boreus et al., 1969). Furthermore, as pointed out by Winckler (1969), differences exist between different species depending on whether the newborn animals remain in the nest like rats and rabbits, or leave the nest early like guinea pigs.

The early appearance and development of fluorescent sympathicoblasts found in the present investigation were quite similar to findings by Enemar et al. (1965) in the chick embryo. Thus, the sympathetic chain and adrenal medulla were formed during a short time period of fetal life in both chick and rat. Also the appearance and location of the first fluorescent cells and the secondary segmentation of the trunk were similar. The chick, however, which is relatively mature at hatching, seemed to have a correspondingly earlier development. The development of segmentation of the trunk is in accordance with the findings in man (see e.g. Lutz, 1968).

Björklund et al. (1968) mentioned that fluorescence could be observed in sympathetic ganglia even in the 12 day mouse embryo. This would be somewhat earlier than in the rat, and may be explained by the fact that the mouse embryo at this stage is in fact both heavier and larger than the rat embryo, although the rat is 4 times as heavy as the mouse at birth (Schumann, 1969).

The origin of the different groups of sympathicoblasts has been much discussed As shown by Enemar *et al* (1965) for the chick, the fluorescence technique might contribute significantly to this problem and this holds true also for mammals A close follow up of the early developmental stages which is beyond the scope of the present investigation, would then be needed

The relatively high fluorescence intensity and large diameter of outgrowing axons, as compared to adult non terminal axons, is in accordance with earlier findings An increased axonal fluorescence intensity was noted by Enemar *et al* (1965) and by Friedman *et al* (1968) in the developing heart, and by Ehinger *et al* (1968) in the human fetus The relatively large diameter of such axons in the heart has also been described (Schiebler and Heene 1968, Winckler 1969) An increased fluorescence intensity of outgrowing axons can be demonstrated also in the central nervous system (Maeda and Dresse 1968), and similar findings have been noted in tissue cultures of sympathetic ganglia (Sano *et al* 1967)

The finding of fluorescence in the fetal pigment epithelium of the eye is in accordance with earlier investigations (Ehinger and Rosengren 1967 Winckler and Turner 1969)

Postnatal observations

The sympathetic adrenergic innervation of the adult rat which provided the basis of comparison in the present investigation has been extensively studied with the fluorescence technique (see Norberg 1967) Comparatively few studies on the post-natal development have been published

The early appearance of the mouse iris was briefly described by Olson (1967) and is consistent with the present observations on rats

The development of the innervation of the heart has been studied in the rat by Schiebler and Heene (1968) in the guinea pig by Winckler (1969) and in the rabbit by Friedman *et al* (1968) On the whole our results confirm these observations However, Schiebler and Heene (1968) did not observe the increased axonal fluorescence, and did not detect any major differences between the 1 and 12 day old animals This discrepancy is probably due to difficulties encountered in the cryostat-sectioning technique employed by these authors (Heene 1968) since reaction with dry paraformaldehyde was needed (*cf* Hamberger 1967) In the present investigation a plexus of fluorescent nerves was observed at the 12th day in contrast to the few fibres found at birth and after both 1 and 12 days with the cryostat technique

In the submaxillary gland the development of the adrenergic nerves parallelled the maturation of the acini The difference between the non innervated [illegible] and the innervated submaxillary gland (Norberg and Olson 1965) was [illegible]

The ingrowth of nerves observed in the vas deferens is in accorda[illegible] electronmicroscopical studies of Yamauchi and Burnstock (1969) in [illegible] ingrowth from the periphery of an organ was noted also in the hear[illegible] described also for *e g* the corpus pineale (Machado *et al* 1967)

The small i[illegible] a well developed meshwork of fluor[illegible]

plexus of Auerbach soon after birth, when the other tissues examined contained only few nerves. A direct innervation of the circular muscle layer appeared later.

The innervation of the intestine of the newborn guinea pig (Gabella and Costa 1968) seems to be even more rapid, with an innervation of the circular muscle layer already in the 1 day old animal.

Observations concerning the enterochromaffin cells are in accordance with the findings of Håkanson *et al.* (1969).

Iversen *et al.* (1967) described the concentrations of the endogenous NA in different organs (heart, salivary glands and intestine) of the developing rat. They found NA in all organs on the first postnatal day (the earliest stage examined) which is correlated to the present findings of fluorescent nerves in these organs at that time. Quantitatively, the endogenous levels are very well correlated to the relative amount and intensity of the fluorescent fibres found (*cf.* Olson *et al.* 1968). The low NA level in the heart corresponds to the few moderately fluorescent fibres in the atrium. The somewhat higher NA levels in the salivary glands and in the intestine can easily be explained by the large amount of strongly fluorescent non-terminal axons and by the great number of varicose terminals in the plexus of Auerbach respectively.

It was found (Iversen *et al.* 1967) that the concentration of the endogenous NA increased up to the 42nd day. This indicates that there was either an increase in the amount of nerves per gram tissue and/or an increase in the concentration of NA in the nerves. The present study favours an increase of the number of nerves in the heart while the latter alternative seems relevant for the salivary glands and the intestine since the density of nerves seemed to decrease during postnatal growth of these organs (*cf.* Sachs *et al.* 1970). This is furthermore supported by the fact that the NA concentration of the developing nerves seems to be lower than in adult nerves as their fluorescence intensity was lower.

The development of the adrenergic nerves in the iris and atrium is well correlated to the development of neuronal uptake of 3H-NA in these organs (Sachs *et al.* 1970).

This investigation was supported by grants from the Swedish Medical Research Council (B70-14X 711 05B and B70 14X 714 05) and from "Ollie och Elof Ericssons Stiftelse", "Svenska Livförsäkringsbolags Nämnd för Medicinsk Forskning" and "Stiftelsen Therese och Johan Anderssons Minne".

The skilful technical assistance of Mrs Ulla Flyger and Mrs Barbro N[illegible] is gratefully acknowledged.

For generous supplies of drugs the authors are indebted to Swedish Ciba AB, Göteborg, Sweden (Serpasil®) and Pfizer, Stockholm, Sweden (Niamid®).

References

Bjorklund, A., A. Enemar and B. Falck: Monoamines in the hypothalamo-hypophyseal system of the mouse with special reference to the ontogenetic aspects. *Z. Zellf[illegible]* 1968, *89*, 590–[illegible]

Dr[illegible] adrenergic [illegible] physiol. [illegible]

Corrodi H and G Jonsson The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines A review on the methodology *J Histochem Cytochem* 1967 *15* 65—78

Ehinger B and E Rosengren DOPA in ocular pigment cells in mammalian albinotic embryos *Experientia* (Basel) 1967 *23* 411

Ehinger B G Gennser Ch Owman and H Persson Histochemical and pharmacological studies on amine mechanisms in the umbilical cord umbilical vein and ductus venosus of the human fetus *Acta physiol scand* 1968 *72* 15—24

Enemar A B Falck and R Håkanson Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo *Develop Biol* 1965 *11* 268—283

Falck B Observations on the possibilities of the cellular localization of monoamines by a fluorescence method *Acta physiol scand* 1962 *56* Suppl 197 1—25

Falck B N A Hillarp G Thieme and A Torp Fluorescence of catecholamines and related compounds condensed with formaldehyde *J Histochem Cytochem* 1962 *10* 348—354

Friedman W F P E Pool D Jacobowitz S C Seagren and E Braunwald Sympathetic innervation of the developing rabbit heart *Circulat Res* 1968 *23* 25—32

Gabella G and M Costa Le fibre adrenergiche nel canale alimentare *Accad med* 1967 *130* 1—12

Gabella, G and M Costa La distribuzione delle fibre adrenergiche nell intestino tenue di cavia neonata Scr med in onore del Prof G Dellepiane *Accad med* 1968 453—455

Håkanson R Ch Owman and N O Sjöberg Three different systems of monoamine storing cells in the gastrointestinal tract of fetal and neonatal rats *Acta physiol scand* 1969 *75* 213—220

Hamberger B Da . . . in isolated tissues of the rat A . 1—56

H . und 5 hydroxytryptamine am

Iversen L L J de Champlain J Glowinski and J Axelrod Uptake storage and metabolism of norepinephrine in tissues of the developing rat *J Pharmacol exp Ther* 1967 *157* 509—516

Jonsson G and G S . . ion of ^{3}H noradrenaline in adrenergic nerves of . monoamine oxidase and tyrosine hydroxylase inh 357

Lutz . und des Nervus Vertebralis *Z Anat Ent* wic

Mach . . Bragg and A B M Machado A histochemical study of sympa het . .

M . .

Malmfors T Studies on adrenergic nerves Thus use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 *64* Suppl 248 1—93

Norberg K A Transmitter histochemistry of the sympathetic adrenergic nervous system *Brain Res* 1967 *5* 125—170

Norberg K A and L Olson Adrenergic innervation of the salivary glands in the rat *Z Zellforsch* 1965 *68* 183—189

Olson L Outgrowth of sympathetic adrenergic neurons in mice treated with a Nerve Growth Factor NGF *Z Zellforsch* 1967 *81* 155—173

Olson L B Hamberger G Jonsson and T Malmfors Combined fluorescence histochemistry and ^{3}H noradrenaline measurements of adrenergic nerves *Histochemie* 1968 *15* 38—45

Olson L Intact and regenerating sympathetic noradrenaline axons in the rat sciatic nerve *Histochemie* 1969 *17* 349—367

Olson L and L Ungerstedt A simple high capacity freeze drier for histochemical use *Histochemie* 1970 *22* 8—19

Read J B and G Burnstock A method for the localization of adrenergic nerves during early development *Histochemie* 1969 *20* 197—200

Richardson K Electron microscopic identification of autonomic nerve endings *Nature* Lond) 1966 *210* 756

Sachs Ch J de Champlain T Malmfors and L Olson Postnatal development of noradrenaline uptake in adrenergic nerves *In vitro* isotope studies of different rat tissues with or without pretreatment with drugs *Europ J Pharmacol* 1970 *9* 67—79

SANO, Y, G ODAKE and T YONEZAWA, Fluorescence microscopic observations of catechol- [illegible]

[illegible] der [illegible] ise- [illegible] ing

WINCKLER, J and H B TURNER, Über das Vorkommen von Katecholaminen im Pigmentepithel des Meerschweinchenauges während der Entwicklung *Histochemie* 1969 *19* 272—280

YAMAUCHI, A and G BURNSTOCK, Postnatal development of the innervation of the mouse vas deferens A fine structural study *J Anat* (Lond) 1969 *104* 17—32

Acta physiol. scand. 1970. 80. 289—294

From King Gustaf V's Research Institute, Stockholm and the Department of Geriatrics, Uppsala University, Sweden

Effect of Prostaglandin E_1 on Fat Mobilizing Lipolysis in Rat Adipose Tissue in Relation to the Nutritional Condition

By

LARS A CARLSON and HORACE MICHELI[1]

Received 29 January 1970

Abstract

CARLSON, L A and H MICHELI *Effect of prostaglandin E_1 on fat mobilizing lipolysis in rat adipose tissue in relation to the nutritional condition* Acta physiol scand 1970 *80* 289—294

The effect of prostaglandin E1 (PGE_1) on fat mobilizing lipolysis in rat adipose tissue was studied in relation to the nutritional condition by following the release of glycerol during in- [illegible]

(average of 7 expts) These average decreases were not statistically significant Refeeding [illegible] ity of adipose tissue to [illegible] thus of importance for [illegible] the tissue sensitivity to [illegible]

Prostaglandin E_1 (PGF_1) is a potent inhibitor of lipolysis in adipose tissue (Bergstrom *et al* 1968) However it has been observed in two laboratories (Bergstrom and Carlson 1965, Stock and Westermann 1966) that PGE_1 does not inhibit lipolysis in vitro in adipose tissue taken from fasted rats In contrast from these reports Kupiecki (1967) found that PGE_1 was an effective antilipolytic agent both in vivo and in vitro in fasted rats In this study we have reexamined this question and studied the effect in vitro of various doses of PGE_1 on adipose tissue from fed and fasted rats

Material and methods

Male Sprague Dawley strain rats (A B Anticimex, Sollentuna Sweden) weighing 145—190 g were used after a stay of at least three days in our animal department They were fed with Anticimex rat pellets The studies were started around 10 a.m by killing the rats *Fed* ra[illegible] had free access to food and water Food was taken away around 9—10 a m the day before study for *fasted* rats but they had free access to drinking water Twelve animals were gui[illegible] ed in each experiment and the epididymal fat pads were cut randomized in flasks (P[illegible]

[1] Visiting scientist from the University of [illegible] Clinique Medicale, Hopital [illegible] supported by the Fonds National Suisse de la [illegible] scientifique, Berne, Switze[illegible]

n 1965) if not stated otherwise
ffer with 2 % human albumin
7 4, were stoppered and stayed

determination of gl
glandin E_1 (PGE_1)
tion All the flasks h
in the experiment
Institutet, Stockholm
which was kept at −17 C up to 4 months Dilutions in saline were prepared less than 1 hr before use at room temperature Statistical methods were according to Snedecor (1956)

Results

Effect of PGE_1 in the fed state

The effect of different doses of PGE_1 on glycerol release is given in Table I and a statistical evaluation of comparisons of the values is presented in Table II The lowest dose, 0 001 μg/ of PGE_1 per ml incubation medium, lowered the glycerol release in one of 2 expts The next dose, 0 01 μg, and all higher doses in each experiment had a significant inhibitory effect on lipolysis when compared to the basal lipolytic rate PGE_1 maximally inhibited lipolysis at the concentration of 0 1 μg per ml At this dose level the mean lipolytic rate was 41 per cent of the basal rate With 1 μg per ml the inhibition of glycerol release was less marked than with 0 1 and here the average lipolytic rate was 48 per cent of the basal However the difference between the values for these two dose levels within one and the same experiment was not significant (Table II) On the other hand with 10 μg per ml the glycerol values were always significantly higher than those seen with 0 1 μg/ml (Table II) although they were lower than the values for the basal release On a percentage basis the lipolytic rate observed with 10 μg of PGE_1 was 68 per cent of the basal rate The parallel appearance of the dose response curves from the six experiments is evident from Fig 1

TABLE I Effect of different doses of PGE_1 on the glycerol release from adipose tissue of fed rats Mean values standard error of the mean and within brackets number of incubation flasks

PGE_1 μg/ml	0	0 001	0 01	0 1	1	10
Exp no	Glycerol release μmoles/g/hr					
1	2 21 ± 11 (7)		1 47 ± 12 (7)	0 77 ± 07 (6)	0 92 ± 17 (6)	1 28 ± 08 (5)
2	1 77 ± 04 (7)	—	1 13 ± 05 (7)	0 84 ± 06 (6)	0 93 ± 05 (6)	1 31 ± 07 (6)
3	2 01 ± 14 (6)	1 50 ± 14 (5)	1 13 ± 17 (5)	0 73 ± 05 (5)	0 85 ± 13 (5)	1 47 ± 05 (6)
4	1 94 ± 11 (6)	1 85 ± 12 (6)	1 38 ± 13 (5)	0 85 ± 02 (5)	0 97 ± 09 (5)	1 35 ± 08 (5)
5	2 38 ± 07 (6)	— (6)	1 48 ± 12 (6)	1 09 ± 05 (6)	1 22 ± 07 (6)	1 73 ± 09 (7)
6	2 73 ± 06 (5)		—	1 02 ± 13 (5)	—	1 64 ± 09 (6)
Average in % of basal	100	85	64 ± 3	41 ± 2	48 ± 2	68 ± 3

TABLE II P value for the statistical significance of the difference between the glycerol values for different doses of PGE_1 in Table I P is smaller than the figure given ns — not significant ($P > 0\ 05$)

Compared doses from Table I	0—0 01	0—0 1	0—1	0—10	0 1—1	0 1—10
Exp no						
1	0 001	0 001	0 001	0 001	ns	0 001
2	0 001	0 001	0 001	0 001	ns	0 01
3	0 01	0 001	0 001	0 01	ns	0 001
4	0 05	0 001	0 001	0 01	ns	0 001
5	0 001	0 001	0 001	0 01	ns	0 001
6	—	0 001	—	0 001	—	0 01

Effect of PGE_1 in the fasted state

The effect of different doses of PGE_1 on glycerol release is given in Table III and a statistical evaluation of comparisons of the values is presented in Table IV The response of adipose tissue from fasted rats to PGE_1 at different concentrations varied from experiment to experiment and the dose response curve did not have the consistent appearance seen in dose response curves obtained from fed rats (Fig 1)

In 4 of the 7 expts with fasted rats significant inhibition was observed with two or more doses of PGE_1 In the remaining three experiments statistically significant inhibition was not found with any dose of PGE_1 It should be stressed however that in the instances where inhibition was seen this inhibition was small compared to that obtained in the fed state As a mean of the 7 expts in fasted rats the inhibition was most marked with 1 μg per ml of PGE_1 but this reached only 86 ± 7 per cent of the basal glycerol value in contrast to the mean obtained in the studies with fed rats

TABLE III Effect of different doses of PGE_1 on the glycerol release from adipose tissue of 24 hour fasted rats Mean values ± standard error of the mean and within brackets number of incubation flasks

PGE_1 μg/ml	0	0 01	0 1	1	10
Exp no	Glycerol release μmoles/g h				
1	1 98 + 06 (7)	1 70 + 07 (7)	1 71 ± 17 (6)	1 49 + 15 (6)	1 66 + 13 (6)
2	0 96 08 (6)	0 94 09 (5)	0 93 ± 06 (5)	0 80 + 06 (5)	0 89 ± 05 (6)
3	1 86 06 (6)	1 49 + 08 (5)	1 60 10 (5)	1 79 + 16 (5)	1 68 ± 11 (5)
4	1 63 + 07 (7)	—	1 33 ± 03 (6)	1 27 ± 03 (6)	1 36 ± 05 (6)
5	1 64 ± 04 (7)	—	1 25 ± 13 (5)	0 95 ± 10 (6)	1 57 ± 14 (5)
6	0 88 + 04 (7)	—	1 00 + 11 (6)	0 83 ± 08 (6)	0 90 ± 08 (6)
7	1 70 + 14 (6)	1 59 + 18 (5)	1 68 + 15 (5)	2 00 ± 23 (5)	2 04 ± 23 (5)
Average in % of basal	100	90 ± 4	91 ± 5	86 ± 7	95 ± 5

TABLE IV P-values for the statistical significance of the difference between the glycerol values for different doses of PGE_1 in Table III P is smaller than the figure given ns = not significant (P > 0 05)

Compared doses	0—0 01	0—0 1	0—1	0—10	0 1—1	0 1—10
Exp no						
1	0 05	ns	0 05	0 05	ns	ns
2	ns	ns	ns	ns	ns	ns
3	0 01	0 05	ns	ns	ns	ns
4	—	0 01	0 001	0 01	ns	ns
5	—	0 05	0 001	ns	ns	ns
6	—	ns	ns	ns	ns	ns
7	ns	ns	ns	ns	ns	ns

where the glycerol release was 48 ± 2 per cent of basal with 1 μg per ml of PGE_1 and 41 ± 2 per cent with 0 1 μg per ml

While in all experiments with fed rats there was a significant increase in the glycerol release when the dose of PGE_1 was increased from 0 1 to 10 μg per ml this phenomenon was not seen in any experiment with fat from fasted rats The basal lipolytic rate was unusually low in 2 of the 3 expts where PGE_1 had no inhibitory effect at any concentration (expt 2 and 6 Table III) To see if the basal lipolytic rate was of importance for the inhibitory action of PGE_1 we have plotted results obtained in 16 different experiments with 1 μg of PGE_1 in the left part of Fig 2 In 4 of these 16 expts we obtained a statistically significant inhibition of lipolysis The figure shows that absence of antilipolytic effect occurred when the basal lipolytic rate was high as well as low

Effect of PGE_1 in fasted, glucose fed rats

The effect of glucose given intragastrically on the response to PGE_1 is shown in Table V in two experiments where simultaneously untreated and saline treated fasted rats were studied When the rats were either not treated or when they received saline one hour before death there was a small but not significant decrease of glycerol release with the addition of 1 μg per ml of PGE_1 to the incubation medium In contrast when the rats were given 1 g of glucose one hour before death PGE_1 significantly in

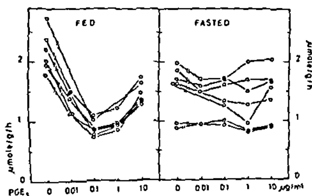


Fig 1 Comparison of the effect of different doses of PGE_1 on basal glycerol release from adipose tissue of fed and fasted rats

TABLE V Effect of glucose on the response of adipose tissue from 24 hour fasted rats to PGE_1 in vitro

Treatment Addition in vitro	None		NaCl		Glucose	
	0	PGE_1	0	PGE_1	0	PGE_1
Exp no	Glycerol release, μmole/g/hr					
1	2 09 ± 05 (6)	2 02 ± 04ns (6)	1 92 ± 08 (6)	1 70 ± 12ns (6)	2 09 ± 09 (6)	1 57 ± 08** (6)
2	1 67 ± 11 (6)	1 57 ± 09ns (6)	1 52 ± 03 (6)	1 36 ± 09ns (6)	1 57 ± 03 (6)	0 99 ± 07*** (6)

PGE_1 = prostaglandin E_1, 1 μg ml NaCl = 0 9 % solution, 2 ml GLUCOSE = 50 % solution, 2 ml ns = no significant effect **, *** = effect significant at $P < 0\ 01$ and $< 0\ 001$ respectively

In each experiment twelve rats fasted 24 hours, were randomized into three groups of 4 rats given different treatment One group had no treatment, one received saline and one glucose by stomach tube 1 hour before killing

hibited glycerol release The effect of glucose and saline intragastrically has been studied altogether in 5 and 7 expts respectively (Fig 2) The adipose tissue of the fasted rats treated with glucose had a significant reduction of the glycerol release in response to PGE_1 On the average the glycerol release was reduced to 68 ± 3 per cent of the basal value On the other hand in NaCl treated or untreated fasted rats 3 of 7 and 4 of 16 expts respectively showed a significant effect of PGE_1 (Fig 2) The average glycerol release in these two groups after PGE_1 was 85 ± 5 and 89 ± 3 per cent of basal

Discussion

The effect of PGE_1 on adipose tissue lipolysis in vitro is clearly related to the nutritional condition tissues from fed rats beeing sensitive while tissues from fasted animals are either unsensitive or less sensitive to PGE_1 We have no explanation for this effect of fasting Several possibilities can be discussed, however Fasting may decrease the "availability" of the structures sensitive to PGE_1 Fasting might change the lipolytic processes so that they are no longer sensitive to PGE_1 This could *e g* be ana

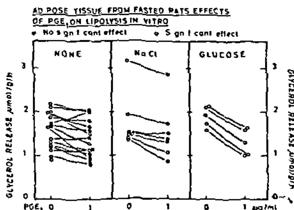


Fig 2 Effect of PGE_1 on basal glycerol release from adipose tissue of rats fasted 24 hrs To the left 16 expts where the rats were studied as described under Material and methods In the middle 7 expts where the rats were given saline intragastrically and to the right 5 expts where the rats were given glucose 1 hr before killing Black dots indicate that the effect of PGE_1 was insignificant ($P > 0\ 05$) while open dots indicate that PGE_1 had significantly inhibited the glycerol release

loguous to the situation in rabbit adipose tissue where PGE_1 did not inhibit basal lipolysis (Micheli 1969) In this tissue it was suggested that the unsensitivity of basal lipolysis to PGE_1 was due to lack of (or low) formation of cyclic AMP In accordance with this basal lipolysis was not inhibited in the adipose tissue of the rabbit by nicotinic acid (Boberg *et al* 1969), which compound is also known to inhibit tissue accumulation of cyclic AMP (Butcher *et al* 1968) However, nicotinic acid inhibits lipolysis in adipose tissue of the fasted rat (Bergstrom and Carlson 1965) The effect of fasting on adipose tissue levels of cyclic AMP or on the adenyl cyclase and the phosphodiesterase systems are not known

In vivo the mobilization of fat from adipose tissue is increased during fasting This may partly be due to increase in the fat mobilizing lipolysis The cause(s) of this increase are unknown They may involve changes in different physiologic mechanisms such as increases in processes stimulating and/or decreases in processes inhibiting lipolysis The development of unsensitivity of adipose tissue to the antilipolytic effect of PGE_1 during fasting may be of physiological importance in enhancing fat mobilizing lipolysis in fasting

It is of interest that the effect of PGE_1 on lipolysis is still more changed in diabetic adipose tissue In adipose tissue from rats treated with anti insulin serum PGE_1 stimulated glycerol production (Carlson and Micheli 1969) This effect and the present finding that feeding of glucose rapidly restores the sensitivity of adipose tissue to PGE_1 suggests that insulin may be required if PGE_1 should have an antilipolytic effect The fact that the incubation medium always contained glucose makes it unlikely that it is the glucose per se which restored the sensitivity of the tissue to PGE_1

Supported by grants from the Swedish Medical Research Council (19X 204 06)

References

BERGSTROM S and L A CARLSON Influence of the nutritional state on the inhibition of lipolysis in adipose tissue by prostaglandin E_1 and nicotinic acid Prostaglandin and related factors 46 *Acta physiol scand* 1965 *65* 383—384

BERGSTROM S L A CARLSON and J R WEEKS *The prostaglandins A family of biologically active lipids* *Pharmacol Rev* 1968 *20* 1—48

BIZZI A and L A CARLSON Stimulatory effect of ethanol on glycerol release from rat adipose tissue *in vitro* *Life Sci* 1965 *4* 2123—2128

BOBERG J H MICHAELI and L RAMMER Effect of nicotinic acid on ACTH and noradrenaline stimulated lipolysis in the rabbit II In vitro studies [illegible] PGE_1 *Acta physiol scand* 1970 *79* 299—304

BUTCHER R W C E BAIRD and E W SUTHERLAND Effect of lipolytic and antilipolytic substances on adenosine3 5 monophosphate levels in isolated fat cells *J biol Chem* 1968 243 1705— 1 1

CARLSON L A and H MICHELI *Paradoxical effect of prostaglandin E_1 in vitro on lipolysis in* [illegible] Congress Intern Diabetes Feder Stock

itty acids in the

of noradrenaline
9—298
956
Sw ca
St 66

Acta physiol. scand. 1970. 80. 295—298

From the Department of Animal Nutrition, Genetics and Hygiene, Royal Veterinary College, and the Institute of Biochemistry, University of Stockholm, Sweden

The Relationship between Arylesterase Activity and Growth in Rats

By

KLAS BERTIL AUGUSTINSSON and BENGT HENRICSON

Received 6 February 1970

Abstract

AUGUSTINSSON, K.-B. and B. HENRICSON. *The relationship between arylesterase activity and growth in rats.* Acta physiol. scand. 1970. *80*. 295—298.

There was a highly significant negative correlation between blood plasma arylesterase activity and growth rate in male rats when the enzyme activity was determined at younger ages (11—32 days). In females the correlation was only vaguely indicated. A direct or indirect causal relationship between the two parameters is discussed.

In a series of experiments various causes of variation of the blood plasma arylesterase activity level in rats have been analyzed. The influence of sex, age and condition of momentary food consumption is well established (Augustinsson and Henricson 1965). The additive genetic part of individual variation is probably relatively small (Henricson and Augustinsson 1967). Emotional stress (Augustinsson and Henricson 1969) and the parenteral application of estradiol benzoate greatly influences the level of activity (Augustinsson and Henricson 1970).

Parallel to the various experiments the growth rate of the rats has been routineously monitored. When comparing the enzyme activity level and the growth rate a tendency to a non random correlation was found.

An experiment was then designed and performed more directly to study the growth and food consumption in relation to the esterase activity. The two kinds of experiments will be called A- and B-experiments respectively.

Materials and methods

Wistar rats were used in all experiments. They were given a standardized diet containing 68.5 % wheat starch, 25 % dried skim milk, 1.5 % fodder yeast, 2.5 % olive oil, 0.5 % A.D. mixture and 3 % salt mixture.

In all experiments with the exception of the last the food was given dry, but as this made it difficult to measure the magnitude of consumption (because of wastage) the food was with some water in the last experiment.

Food and water were given *ad libitum*.

TABLE I Correlation between arylesterase activity (ArE) and growth rate

Exp No	ArE detn at age (days)	% gain of weight between ages (days)	Sex	n	Correlation coeff	P
	60 and 90 (mean)	60—90	♂	76	—0 167	0 2 ~ P > 0 1
	"	"	♀	53	—0 230	≃0 1
2	55	40—120	♂	24	—0 251	0 3 > P > 0 2
2		"	♀	20	—0 301	~0 2
3	90 and 100 (mean)	30—100	♂	26	0 186	0 4 > P > 0 3
3			♀	19	0 179	0 5 > P > 0 4
4	65	65—100	♂	59	—0 214	≃0 1
4			♀	69	—0 006	> 0 9

growth time control also varies day of life and ended at the age of

h a manometric technique using phenyl acetate as the substrate (Augustinsson and Henricson 1965) It was determined at the ages indicated in Table I and II

Results

A experiments

Table I summarizes the results of four experiments where the relationship between arylesterase and growth was not the primary interest In three of the four experiments the correlation between arylesterase activity and percental gain of weight is negative but in exp 3 it is positive In no case however is the correlation coefficient significant It should be pointed out that in exp 3 the enzyme activity was measured at the highest age

TABLE II Correlation between arylesterase activity (ArE) at different ages and growth rate between 11 and 80 days of age or growth per unit food between 28 and 80 days

ArE-detn at age (days)	Sex	n	Correlation coeff between ArE and growth rate growth per unit food		P	
			a	b	a	b
11	♂	29	0 865	—0 773		0 001
21	♂	[illegible]	0 810	—0 832		0 001
32	♂	[illegible]	0 678	—0 937		0 001
80	♂	[illegible]	—0 247	—0 487	~0 2	0 01 P > 0 001
11	♀	[illegible]	0 225	0 287	0 2 P > 0 1	0 1 > P > 0 05
20	♀	[illegible]	0 255	0 003	~0 1	0 9
32	♀	39	0 174	0 018	~0 3	~0 7
53	♀	39	0 237	0 089	0 2 > P > 0 1	~0 6
80	♀	39	0 258	0 077	~0 1	0 7 > P ~ 0 6

B-experiment

Growth rate and gain of weight per unit food during the rearing period was correlated to the enzyme activity at different ages

The results (Table II) are very indicative of a negative correlation between the arylesterase activity level and the growth rate especially at younger ages This is valid for males but only vaguely indicated in females

Discussion

In the experiments where arylesterase activity was determined at a relatively great age correlation to growth rate was negative in three out of four experiments The correlation coefficient was in no case significant. However, when measured at a younger age correlation becomes highly significant in males This is in good agreement with the fact that the arylesterase activity in rats seems to be very much influenced by environmental factors (Henricson and Augustinsson 1967) At younger ages the enzyme activity is consequently more related to the animal s own constitution The same should to a certain extent also be valid for growth rate

There are al o certain indirect indications of a negative correlation between the arylesterase activity level and growth Thus females have a higher enzyme activity level than males (Augustinsson and Henricson 1965) and the growth rate in females is considerably slower than in males During starvation the arylesterase activity level rises markedly (Augustinsson and Henricson 1965) Starvation of course results in a retardation of growth

Shellhammer (1961) reported on a negative correlation between the levels of acetylcholinesterase of the brain and the body weight in two strains of wild mice As there was no closer knowledge of the age of the animals the correlation could however concern age rather than weight

The negative correlation between arylesterase activity measured in the growing rat and the growth rate which is highly significant in males could be explained in different ways This does not necessarily mean that there is a direct causal connection between the enzyme activity and growth Both could be influenced by a third factor One such common factor could be hormones It is known for instance that estrogens have a considerable effect on both parameters (Meites 1949 Augustinsson and Henricson 1970)

Very little is known about the physiological effects of arylesterase According to a theory (Augustinsson and Henricson 1965) it might mediate the trans-esterification between free fatty acids and a blood plasma albumin like component The utilisation of fatty acids in the metabolism influencing growth might then be related to the level of arylesterase activity in a way which is not clear at the present time

A difference between the sexes concerning the correlation in question is obvious There is some experimental evidence (Augustinsson and Henricson 1970) * arylesterase activity is more heavily influenced in males than in females F

of estradiol benzoate If the correlation between enzyme and growth has direct causal nature the involvement of the enzyme in the growth process should be less pronounced in females than in males

This investigation was supported by grants from the Swedish Medical Research Council and the Swedish Natural Science Research Council We are indebted to Mrs Inga Andersson and Mr Erik Johansson for skilled technical assistance

References

AUGUSTINSSON K B and B HENRICSON Some factors regulating normal arylesterase activity of blood plasma in rats *Acta physiol scand* 1965 *64* 418—425

AUGUSTINSSON K B and B HENRICSON Stress induced alterations in arylesterase activity in rats *Biochem Pharmacol* 1969 *18* 21—27

AUGUSTINSSON K B and B HENRICSON Sex hormones as a factor controlling the blood plasma arylesterase in rats *Experientia* (Basel) In press

HENRICSON B and K B AUGUSTINSSON Causes of individual variation in arylesterase activity in blood plasma in rats *Hereditas* 1967 *57* 178—184

MEITES J Relation of food intake to growth-depressing action of natural and artificial estrogens *Amer J Physiol* 1949 *159* 281—286

SHELLHAMMER H S Variation in brain acetylcholinesterase in the wild mice *Mus musculus* and *Reithrodontomys megalotis* *Physiol Zool* 1961 *34* 312—318

Acta physiol. scand. 1970. 80. 299—306

From the Department of Pharmacology, Karolinska institutet, Stockholm, Sweden

The Effect of Glucose on Histamine Release from Isolated Rat Peritoneal Mast Cells Induced by Adenosine-5'-Triphosphate

By

B. Diamant and C. Peterson

Received 21 February 1970

Abstract

Diamant, B. and C. Peterson. *The effect of glucose on histamine release from isolated rat peritoneal mast cells induced by adenosine-5'-triphosphate.* Acta physiol. scand. 1970. *80.* 299—306.

Histamine release from isolated rat peritoneal mast cells induced by ATP in an aerobic milieu increased in the presence of glucose. Irrespectively whether the cells were exposed to glucose prior to ATP or the two agents were added together to cells, optimal histamine release was noted with 0.3—0.5 mM of glucose and amounted to 1.5—2 times that observed in the absence of glucose. In contrast, under similar conditions compound 48/80-induced histamine release was not affected by glucose. The velocity of ATP-induced histamine release and the final amount of histamine released within 30 min of incubation increased in the presence of glucose. A lag period of about 1 min was noted before histamine release occurred when initiated by ATP (3.9×10[illegible] M). This lag period was not changed by glucose. Iodoacetate inhibited histamine release induced by ATP as well as by compound 48/80. The glucose-activated part of ATP-induced histamine release seemed especially sensitive to iodoacetate. The results suggest that glucose through its metabolism in the cells exerts an enhancing effect on ATP induced histamine release.

The mechanism of histamine release from isolated rat mast cells induced by ATP differs in several respects from that of compound 48/80. However, the dependence on available endogenous energy for the histamine release to occur seems to be a common feature for both releasing agents (Diamant and Krüger 1967).

When histamine release from isolated rat mast cells is induced by compound 48/80 there seems to be an energy dependent extrusion of histamine containing granules through the cell membrane followed by a passive exchange between histamine of the extruded granules and cations in the surrounding medium (Thon and Uvnäs 1967). The evidence for the energy dependence of the reaction is based on the finding that degranulation of rat mesentery mast-cells *in situ* and histamine release from rat lung tissue do not take place in an anoxic milieu or in the presence of metabolic inhibitors (Junqueira and Beiguelman 1955, Diamant and Uvnäs 1961). However, the presence of glucose was found to completely counteract the effects of anoxia and inhibitors of

oxidative metabolism on the action of compound 48/80 It was therefore suggested that in case of compound 48/80, energy furnished from the Embden Meyerhof pathway would suffice to deliver energy to some energy dependent step in the mechanism leading to degranulation and histamine release (Diamant and Uvnas 1961)

Histamine release from isolated rat mast cells induced by adenosine 5'-triphosphate (ATP) was also found to be inhibited by the presence of metabolic inhibitors such as oligomycin or 2,4 dinitrophenol (Diamant and Kruger 1967) In contrast to the effect of compound 48/80, histamine release induced by ATP could not be restored to the original value by preincubation of the cells with glucose before addition of the inhibitor This indicates that histamine release induced by ATP is more dependent on energy derived from oxidative phosphorylation than that of compound 48/80 (Diamant 1969b)

While investigating the influence of different inhibitors on histamine release from isolated rat mast cells and the effect of glucose on the inhibition, it became apparent to us that in the absence of inhibitors glucose activated histamine release induced by ATP The present study was undertaken to investigate this effect more in detail

Methods and materials

Isolation of rat peritoneal mast cells

Peritoneal mast cells were isolated from male Sprague Dawley rats weight 350 –400 g) in
general following the method of Uvnas [illegible] method
glucose was omitted from all solutions [illegible]
experiment After separation of the m[illegible]
washed 3 times each time with 5 ml of [illegible]
KCl (2 7 mM) $CaCl_2$ (0 9 mM) and [illegible]
with 10 % (v/v) Sorensen phosphate [illegible]
suspended in 360—590 μl of the same solution giving a cell concentration varying between 2 2 × 10^6—3 9 × 10^5 cells/ml in different experiments The cells were counted in a Burker chamber

Incubation procedures

All incubations of the mast cells were carried out in open test tubes at + 37° C and at pH 7 0

Experiments concerning histamine release To glass tubes (volume 10 ml) containing 2 ml of the buffered salt solution (see above) 14 18 μl of the cell suspension was added giving a cell concentration of 1600—2600 cells/ml The mast cells were preincubated for 10 min with and without glucose before the addition of ATP (3—4 × 10 M) or compound 48/80 (0 8 μg/ml) Incubation was stopped by placing the tubes in ice cold water Histamine release at time zero (Fig 2 and 3 a) denotes cells which immediately after addition of ATP were placed in ice cold water The tubes were centrifuged (350 × g) for 10 min at + 4° C The supernatants were decanted into new tubes Two ml of distilled water was added to the residues to release the remaining histamine from the cells

Experiments concerning hydrolysis of exogenously applied ATP To glass tubes (volume
3 ml) con[illegible] 2 0 or 4 9 ×
10^{-5} M) [illegible] reaction was
started by [illegible] centration of
100 000—[illegible] the addition
of NaOH [illegible] 0 C for 10
min Standards containing known concentrations of ATP and blanks were carried through the incubation procedure

Determination of histamine release

Histamine in the supernatants and the corresponding residues was determined according to the fluorescence method of Shore *et al* 1959 omitting the extraction procedure (Bergen

dorff 1965) The histamine release was expressed in per cent of the total histamine content of the cells The spontaneous histamine release ranged between 1—6 % in individual experiments and has been deducted from all values presented Each value represents mean of duplicate samples

Determination of ATP hydrolysis

ATP was determined by the enzymatic fluorescence method of Lowry *et al* (1964) 150 µl

moles of ATP hydrolysed × cell⁻¹ × 2 min⁻¹

Materials

Adenosine 5 triphosphate (ATP), triphosphopyridine nucleotide (TPN) and bovine serum albumin were obtained from Sigma Chemical Company (St Louis, USA) Stock solution of ATP (about 100 mM) was assayed spectrophotometrically pH was adjusted to 7 0 by the use of NaOH Between use the stock solution was stored at −20° C Compound 48/80 was kindly supplied by AB Leo (Halsingborg Sweden) and human serum albumin by AB Kabi (Stockholm Sweden) D(+)glucose (analytical reagent grade) was obtained from Mallinckrodt Chemical Works (St Louis USA) iodoacetic acid from Merck AG (Darmstadt Germany), hexokinase (yeast) and glucose 6-phosphate-dehydrogenase (yeast) from CF Boehringer & Soehne (Mannheim Germany)

Results

The enhancing effect of glucose on histamine release from isolated rat mast cells induced by ATP is demonstrated in Fig 1 The effect was evident already with 0 05 mM of glucose and when tested even 0 03 mM had an enhancing effect although less pronounced Optimal histamine release was observed with 0 3—0 5 mM of glucose Further increase of the glucose concentration did not increase the histamine release Preincubation of the cells for 10 min with glucose before the addition of ATP did not markedly affect the histamine release as compared to the simultaneous addi

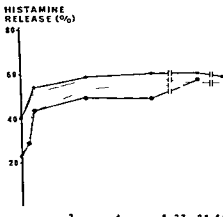


Fig 1 The effect of different concentrations of glucose 0 03—5 8 mM on histamine release from isolated mast cells induced by ATP 3 9 × 10⁻⁵ M) The mast cells were preincubated for 10 min before the addition of ATP The tubes were placed in ice cold water after additional 15 min The results from 2 experiments are shown ☆ ☆ and ○ glucose present during the whole incubation procedure ✦—✦ and ●—● no glucose during preincubation glucose added together with ATP

TABLE I Histamine release from isolated rat mast cells induced by ATP (3 9 × 10^{-4} M) in the presence and absence of glucose The mast cells were preincubated with and without glucose for 10 min before the addition of ATP After additional 15 min the tubes were placed in ice cold water 8 experiments were performed, each in duplicate samples Histamine release is expressed in per cent of the total histamine content of the cells

	no glucose		glucose (0 05 mM)		glucose (0 5 mM)
Histamine release (mean value ± S E)	36 ± 2	$p < 0\ 001$	53 ± 3	$p < 0\ 01$	64 ± 2

tion of glucose and ATP Not shown in the figure is that glucose alone had no releasing activity on the mast cells

The enhancing effect of glucose on ATP induced histamine release was highly significant with 0 05 mM of glucose (Table I) A significant additional increase was found with 0 5 mM of glucose as compared to 0 05 mM (Table I)

In the presence of glucose (0 6 mM) the histamine release induced by ATP rapidly increased during the first 10 min of incubation and reached almost optimal values after 10 min (Fig 2) Preincubation of the cells with glucose for 10 min before the addition of ATP did neither affect the velocity of histamine release nor the amounts released as compared to the simultaneous addition of glucose and ATP

With glucose present histamine release from isolated mast cells induced by ATP occurred at a higher velocity and reached higher values than in the absence of the sugar (Fig 3a and b) Fig 3b demonstrates the effect of glucose on the time course for histamine release induced by ATP (3 9 × 10^{-5} M) during the first 4 min of incubation Under the different experimental conditions the release was linear with time ($r > 0\ 99$) The results indicate that irrespectively of the presence of glucose histamine release started at the same time after a lag period of about 1 min The

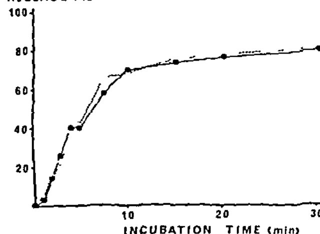


Fig 2 Time course for histamine release from isolated mast cells induced by ATP (3 2 × 10^{-5} M) in the presence of glucose (0 6 mM) The mast cells were preincubated for 10 min before the addition of ATP The result from 1 representative experiment is presented ○——○ glucose present during the whole incubation procedure ●——● no glucose during preincubation glucose added together with ATP

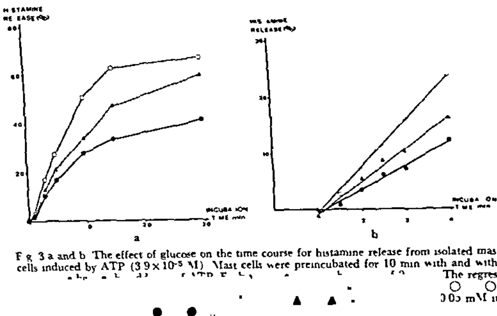


Fig 3 a and b The effect of glucose on the time course for histamine release from isolated mast cells induced by ATP (3.9×10^{-5} M) Mast cells were preincubated for 10 min with and with [illegible] The regres- [illegible] 0 05 mM in [illegible]

velocity of the histamine release increased about 1 4 times with 0 07 mM and about 1 9 times with 0 7 mM of glucose present as compared to the velocity in the absence of glucose

It has been observed that mast cells hydrolyse exogenously applied ATP and a possible correlation between hydrolysis and histamine release has been suggested (Diamant 1969a) It was therefore of interest to investigate if the increased velocity of ATP induced histamine release caused by the presence of glucose could be explained by an enhancement of the hydrolysis of the exogenously applied ATP As evident from table II glucose (0 5 mM) did not influence the hydrolysis of exogenously applied ATP (2.9×10^{-5} M and 4.9×10^{-4} M)

The influence of iodoacetate generally used as an inhibitor of glycolysis on histamine release induced by ATP was investigated in the presence and absence of glucose (0 6 mM), (Fig 4) In the absence of glucose 10^{-5} M of iodoacetate did not significantly influence ATP induced histamine release In the absence of iodoacetate glucose caused an additional 26 ± 2 per cent of the total histamine content to be released (mean ± S E 4 experiments) With 10^{-5} M of iodoacetate present the enhancing effect of glucose was less pronounced and averaged 16 ± 2 per cent The decrease of the effect of glucose in the presence of 10^{-5} M of iodoacetate is statistically significant ($P < 0.05$) With 10^{-4} M of iodoacetate partial inhibition and with 10^{-3} M of iodoacetate almost complete inhibition of the histamine release was found in the absence of glucose Glucose did not enhance histamine release in the presence of iodoacetate in these concentrations

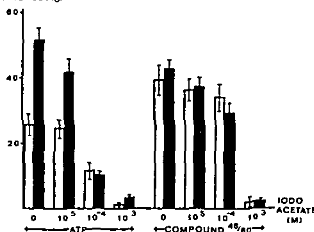


Fig 4 The effect of iodoacetate and glucose (0 6 mM) on histamine release from isolated mast cells induced by ATP ($4 0\times10^{-5}$ M, mean±S E 4 expts) and compound 48/80 (0 8 μg/ml mean±S E , 3 expts) ATP and compound 48/80 were studied together in 3 expts The cells were preincubated with and without iodoacetate for 20 min before the addition of the histamine releasing agents The tubes were placed in ice cold water after additional 15 min Glucose, when present, was added 10 min before the histamine releasing agents Filled bars glucose present Open bars glucose absent

As a comparison the effect of glucose and iodoacetate on histamine release induced by compound 48/80 was also investigated (Fig 4) In contrast to histamine release induced by ATP, glucose did not influence histamine release caused by 48/80 10^{-5} M and 10^{-4} M of iodoacetate did not significantly inhibit 48/80-induced histamine release but 10^{-3} M of iodoacetate caused a pronounced inhibition

Discussion

In earlier reports certain differences have been stressed regarding histamine release induced by ATP and compound 48/80 (Diamant and Kruger 1967, Diamant 1969b) The present investigation further substantiates the difference in action of these agents since the presence of glucose was found not to influence histamine release induced by compound 48/80 in an aerobic milieu whereas a marked enhancing effect was noted on the action of ATP The enhancing effect was apparent already in concentrations that amounted to approximately 0 5 % of that normally found in the blood of the rat (Cole and Harned 1938)

TABLE II Hydrolysis of exogenously applied ATP by isolated rat mast cells in the presence and absence of glucose (10^{-16} moles × cell^{-1} × 2 min^{-1}) 5 experiments were performed each in duplicate samples

	ATP concentration during the incubation			
	$2 9 \times 10^{-6}$ M		$4 9\times10^{-5}$ M	
	glucose (0 5 mM)	no glucose	glucose (0 5 mM)	no glucose
ATP hydrolysis (mean value±S E.)	5 1±0 6	5 3±0 6	7 6±0 9	7 9±0 7

Preincubation of the mast cells with glucose was not necessary in order to observe the enhancing effect The same amount of histamine was released and the same time course for the release process was observed when ATP was added together with glucose to the mast cells as when the cells had been incubated with glucose for 10 min prior to the addition of ATP With the concentrations of ATP used (3—4×10^{-5} M) a lag period of about 1 min was noted before histamine release started Within individual experiments the lag period was not affected by the presence of glucose

The velocity by which histamine was released from the cells as well as the actual amount of histamine released within a given time were enhanced by the presence of glucose 0 3—0 5 mM of glucose was found to be optimal in regard to the enhancing effect on histamine release caused by ATP Disregarding the lag period the release seemed to be linear for at least the first 4 min of incubation and the velocity was about twice as high in the presence of 0 7 mM of glucose as in its absence

In earlier reports the results suggested that the hydrolysis of added ATP by the mast cell membrane might be directly coupled with the release of histamine from the cells (Diamant 1969a 1969b) In the present experiments, hydrolysis of added ATP by the cells was not influenced by the presence of glucose whereas histamine release was activated This indicates that additional factors besides the possibly functional role of the hydrolysis of extracellularly added ATP by the mast cell membrane influence the histamine release process

In contrast to the report of Chakravarty (1968) iodoacetate (10^{-3} M) was found to completely block histamine release from mast cells induced by compound 48/80 irrespectively of the presence of glucose The reason for the difference on the effect of iodoacetate in the two investigations is so far not clear The present results correspond however to the observations of Junqueira and Beiguelman (1955) and Hogberg and Uvnas (1960) who found that iodoacetate blocked compound 48/80 induced degranulation of mast cells in pieces of rat mesentery The present results also show that ATP-induced histamine release (and especially the glucose activated part of the release) seems to be more sensitive towards the action of iodoacetate than histamine release caused by compound 48/80

The results suggest that under aerobic conditions glucose exerts a metabolic effect on mast cells which activates the release process of histamine when induced by extracellular ATP

It has been observed that histamine release induced by ATP occurs without notable degranulation of the mast cells (Diamant and Kruger 1968) Light and electrone microscopic studies instead have suggested that extracellular ATP among various morphological changes induces a dissolution of intracellular granules and that an intact cell membrane is maintained (*Diamant and Kruger 1968 Bloom et al 1969*) It has also been observed that the presence of calcium is necessary for ATP to induce the release of histamine (Diamant and Kruger 1967)

It is therefore concluded that an active aerobic glycolysis as well as calcium are both factors involved in the mechanism of histamine release induced by extracellular ATP The functional significance of the two factors remains to be establish

present special attention is paid to their influence on ATP induced morphological changes of the mast cells as well as to their possible significance as coupling factors for the actual passage of histamine from the cell interior

It should be pointed out that the enhancing effect of glucose on ATP induced histamine release under aerobic conditions in the present investigation was not as apparent in an earlier report (Diamant and Kruger 1967), where only 5—10 % activation of the histamine release was observed in the presence of glucose The reason for this quantitative difference in the two investigations is so far not clear The Ficoll density gradient used for the isolation of the mast cells in the earlier experiments contained glucose (2 mg/ml) whereas in the present investigation glucose was completely omitted during the isolation procedure Preliminary experiments have as yet failed to confirm this as being the only reason to the difference

This study was supported by the Swedish Medical Research Council (B69 14X 1004 04) and Karolinska institutet Stockholm Sweden We are indebted to Mrs Kerstin Green for valuable technical assistance

References

BERGENDORFF A Unpubl observation 1965

BLOOM G D B DIAMANT Ö HAGERMARK and M RITZEN The effects of adenosine 5 triphosphate (ATP) on structure and amine content of rat peritoneal mast cells *Exp Cell Res* In press

COLE V V and B K HARNED Diabetic traits in a strain of rats *Endocrinology* 1938 *23* 318—326

CHAKRAVARTY N Further observations on the inhibition of histamine release by 2-deoxyglucose *Acta physiol scand* 1968 *72* 425—432

DIAMANT B The influence of adenosine triphosphate on isolated rat peritoneal mast cells *Int Arch Allergy* 1969 a *36* 3—21

DIAMANT B Histamine release from mast cells induced by ATP *Acta physiol scand* 1969 b Supp 330 p 29

DIAMANT B and P G KRUGER Histamine release from isolated rat peritoneal mast cells induced by adenosine 5 triphosphate *Acta physiol scand* 1967 *71* 291—302

DIAMANT B and P G KRUGER Structural changes of isolated rat peritoneal mast cells induced by adenosine triphosphate and adenosine diphosphate *J Histochem Cytochem* 1968 *16* 707—716

DIAMANT B and B UVNÄS Evidence for energy requiring processes in histamine release and mast cell degranulation in rat tissues induced by compound 48/80 *Acta physiol scand* 1961 *53* 315—329

HÖGBERG B and B UVNÄS Further observations on the disruption of rat mesentery mast cells caused by compound 48/80 antigen antibody reaction lecithinase A and decylamine *Acta physiol scand* 1960 *48* 133—145

JUNQUEIRA L C U and B BEIGUELMAN Studies on the in vitro action of comp und 48/80 a formaldehyde condensation product on rat mast cells *Texas Rep Biol Med* 1955 *13* 69—75

LOWRY O H J V PASSONEAU F X HASSELBERGER and D W SCHULZ Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain *J biol Chem* 1964 *239* 18—30

SHORE P A A BURKHALTER and V H COHN A method for fluorometric assay of histamine in tissues *J Pharmacol exp Ther* 1959 *127* 182—186

[illegible] release two consecutive steps in the [illegible] *scand* 1967 *71* 303—315

[illegible] ast cells *Exp Cell Res* 1959 *18* [illegible]

Acta physiol. scand. 1970. 80. 307—322

From the Department of Histology, Karolinska Institutet, Stockholm, Sweden

Synthesis of Noradrenaline from 3,4-Dihydroxyphenylalanine (DOPA) and Dopamine in Adrenergic Nerves of Mouse Atrium — Effect of Reserpine, Monoamine Oxidase and Tyrosine Hydroxylase Inhibition

By

GOSTA JONSSON AND CHARLOTTE SACHS

Received 3 March 1970

Abstract

JONSSON, G and CH SACHS. *Synthesis of noradrenaline from 3,4 dihydroxyphenylalanine (DOPA) and dopamine in adrenergic nerves of mouse atrium—effect of reserpine, monoamine oxidase and tyrosine hydroxylase inhibition* Acta physiol scand 1970 *80* 307—322

The *in vitro* synthesis of noradrenaline (NA) from 3,4-dihydroxyphenylalanine (DOPA) and dopamine (DA) in the mouse atrium has been investigated, using isotope, subcellular distribu- [illegible] may in part be explained by decreased competition of the endogenous precursor DA for DA β-hydroxylase but also in part by increased storage sites available for newly formed NA

A large dose of H44/68 (the methylester of *α-methyl-p-tyrosine*), a potent inhibitor of tyrosine hydroxylase, causes a marked depletion of the endogenous NA[1] in the adrenergic nerves of rat iris and mouse atrium, as observed both histochemically and biochemically (Jonsson *et al* 1969, Sachs 1970) Exogenously administered NA is readily taken up into the H44/68-depleted nerves by the axon 'membrane pump', [illegible] incorporated into the amine storage granules as in normal nerves (*Jonsson and*

[1] Abbreviations used NA = noradrenaline, [illegible] dopamine, DOPA = 3,4 dihydroxyphenylalanine, MAO = monoamine oxidase, 6-OH [illegible] hydroxydopamine

1969) The adrenergic nerves thereafter display the same fluorescence morphological appearance as normal nerves (Malmfors and Sachs 1968a, Jonsson and Sachs 1969). It was observed, however, that it was not possible completely to replenish the depleted stores with exogenous NA indicating that amine taken up from the external environment does not easily mix with the endogenous NA pool or pools or that H44/68 treatment reduces the storage capacity of the adrenergic nerves. If the latter possibility is correct there might be changes in the NA synthesis in the adrenergic nerves. This prompted us to investigate the *in vitro* synthesis of NA from labelled DOPA and DA after tyrosine hydroxylase inhibition by H44/68. For comparison the effect of reserpine and/or MAO inhibition on the NA synthesis has also been investigated. Parallel fluorescence histochemical and subcellular distribution studies have been performed.

Materials and Methods

Albino mice (male N.M.R.I. 25—30 g b.w.) were used in the experiments. The animals were killed by decapitation under
(1970). These were then im
7.4 for a short period. The

atria were
The tissue
mentioned

Isolation and determination of radioactive DA and NA

Two atria were extracted with 4 ml cool 0.4 N perchloric acid by homogenization in a glass homogenizer with a tightly fitting pestle. The extracts were neutralized to pH 6.5 by addition of 5 N K_2CO_3, centrifuged $10\,000 \times g$ for 10 min and the supernatants chromatographed on Dowex 50W X4 ion-exchange columns (diameter 4.0 mm, height at pH 1: 50 mm) according to Carlsson and Waldeck (1963). The adsorbed amines were eluted with N HCl and two fractions were collected, the first 7 ml containing NA and the following 8 ml containing DA (see results, Fig. 1). The fractions were transferred to scintillation vials, freeze-dried and 1 ml ethanol and 10 ml toluene
liquid scintillation spectrome
tion of a standard amount
overlap of NA into the DA f

was checked in 2 expts. by using long Dowex 50W X4 columns (Diameter 4.0 mm, height at pH 1: 15 cm) which were used for separation of the three compounds NA, DA and normetanephrine (see results, Fig. 2). Before the extracts from atria incubated in ^{3}H-DA or ^{14}C-DOPA were put on the columns 10 μg carrier NA, DA and normetanephrine had been added. The amines were eluted with N HCl and 50 1 ml fractions were collected, which were analyzed for fluorescence (activation 285 mμ, emission 315 mμ) and radioactivity.

Subcellular distribution studies

4—6 atria were after the incubation re-incubated in fresh buffer for 10 min and then homogenized in 4 ml cool 0.25 M sucrose containing 0.005 M phosphate buffer (pH 7.4) and 0.001 M $MgCl_2$ (Potter and Axelrod 1963) for 60 sec. (24 strokes) using a glass homogenizer with a loosely fitting glass pestle (see Jonsson and Sachs 1969). The homogenates were centrifuged $2\,000 \times g$ to obtain the coarse fraction (P_1), its supernatant centrifuged $107\,000 \times g$ for 60 min

. ... S). The radioact... was
4 N
ribed
itains
atant
are considered when evaluating the intraneuronal distribution of the catecholamines (see Jonsson and Sachs 1969). The results are presented as the amount of catecholamine present in the P₂-fraction as a percentage of that in P₂-fraction plus the high speed supernatant, and thus reflects the affinity to the P₂-fraction

$$\left(\frac{P_2}{P_2+S} \times 100\right)$$

Fluorescence histochemistry

The atria were prepared as whole mounts dried and exposed to gaseous formaldehyde of optimum humidity according to the method of Falck and Hillarp for the histochemical demonstration of biogenic monoamines (see Malmfors 1965, Hamberger 1967, Corrodi and Jonsson 1967).

The following substances were used: dl NA 7 ^{3}H HCl (8.8 Ci/mmole, New England Nuclear Corp., Boston, Mass.), dl NA HCl (Calbiochem), dl DA 7 ^{3}H HCl (8.1 Ci/mmole New
^{14}C-DOPA (54.5 mCi/mmole
6-OH DA HCl (H88/32, AB
ne (Serpasil®, Ciba), H44/68
(Niamid®, Pfizer). The substances were calculated as the free base.

Results

Separation of NA, DA and normetanephrine

The ion exchange technique used in the present investigation gives a good separation between NA and DA, although there is a small overlap of each compound which was corrected for in the calculations (see Fig. 1). This method does not, however, give a clear separation between NA, DA and normetanephrine, while 3-methoxytyramine, which is eluted from the Dowex columns after DA, does not significantly interfere. Since there are possibilities for formation of normetanephrine in the experiments performed in this study, the degree of contamination was checked by using long

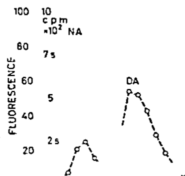


Fig. 1. Ion exchange chromatography on a Dowex 50W X4 column (diameter 4.0 mm, height at pH 1: 50 mm) of an extract of 2 atria from an untreated mouse. The atria had been incubated *in vitro* in 10⁻⁹ M ^{3}H DA for 60 min. Carrier NA (10 µg) and DA (10 µg) had been added to the extract.
●—● Fluorescence (native activation 285 mµ, emission 315 mµ)
— Radioactivity

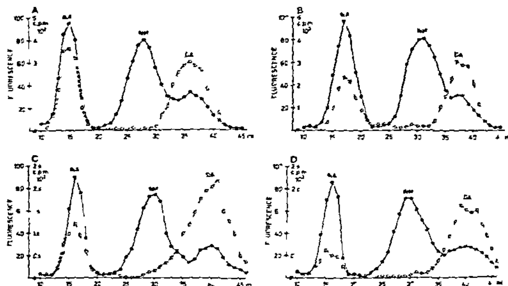


Fig. 2. Ion-exchange chromatographs on Dowex 50W X4 columns (diameter 4.0 mm, height at pH 1 15 cm) of extracts of mouse atria. Carrier NA (10 μg), DA (10 μg) and normetanephrine (NM) (10 μg) had been added to the extracts.
●—● Fluorescence (native activation 285 mμ, emission 315 mμ).
○—○ Radioactivity.
A. Atria from *untreated* mice incubated in 10^{-5} M ^{14}C-DOPA for 60 min.
B. Atria from mice pretreated with *reserpine-nialamide* incubated in 10^{-5} M ^{14}C-DOPA for 60 min.
C. Atria from *untreated* mice incubated in 10^{-5} M ^{3}H DA for 60 min.
D. Atria from mice pretreated with *reserpine-nialamide* incubated in 10^{-5} M ^{3}H DA for 60 min.
In this type of columns 3-methoxytyramine when present is eluted after DA (Rutledge and Jonason 1967).

Dowex ion-exchange columns which allows separation between NA, normetanephrine and DA. From these results it can be concluded that in the experiments presented, very small amounts of normetanephrine are formed since almost all of the radioactivity eluted from the columns was localized in the NA and DA fractions (Fig. 2). These results are also consistent with those of Rutledge and Weiner (1967) who under similar conditions found that the formation of methoxy derivatives was almost negliable (see also Merrills and Offerman 1966). The radioactivity values obtained from the NA and DA fractions respectively from the short Dowex columns thus almost exclusively represent these compounds.

Formation of ^{14}C-DA and ^{14}C-NA from ^{14}C-DOPA

Atria were incubated for 60 min in different concentrations of ^{14}C-DOPA and the amount of ^{14}C-amines recovered from the tissue increased with increasing concentration of ^{14}C-DOPA in the medium (Fig. 3). The mean formation of ^{14}C-NA was 517

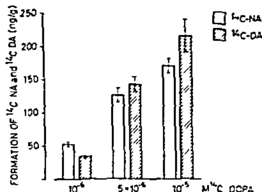


Fig 3 Formation of ^{14}C-NA and ^{14}C-DA in mouse atria from untreated mice incubated *in vitro* for 60 min in various concentrations of ^{14}C-DOPA Each column represents the mean ±S E M of 6—8 determinations

ng/g/hr and of ^{14}C DA 35 2 ng/g/hr for 10^{-6} M ^{14}C-DOPA These values were for 5×10^{-6} M ^{14}C-DOPA in the incubation medium 126 1 (^{14}C-NA) and 142 7 (^{14}C-DA) *ng/g/hr*, and for 10^{-5} *M* ^{14}*C-DOPA* 171 4 (^{14}C-NA) and 215 5 (^{14}C-DA) ng/g/hr

The effect of reserpine (10 mg/kg *i p* 16 hr) and/or nialamide (100 mg/kg *i p* 2 hr) or H44/68 (500 mg/kg *i p* 16 hr) on the formation of ^{14}C NA and ^{14}C-DA are shown in Fig 4 Nialamide pretreatment resulted in a considerably increased formation of both ^{14}C NA and ^{14}C-DA, whereas reserpine caused a marked reduction of ^{14}C amines recovered from the tissue This reduction in ^{14}C-NA formation could be partially counteracted by additional nialamide treatment, while the ^{14}C-DA formation was increased compared to untreated after reserpine+nialamide pretreatment H44/68 treatment produced an increased formation of ^{14}C-NA while the amount of ^{14}C-DA recovered from the tissue was approximately the same as that of untreated When comparing the ^{14}C NA formation after H44/68 pretreatment for 1 hr and 16 hrs respectively, it was found that the shorter time of treatment gave only a slightly increased ^{14}C-NA formation compared to untreated while the longer time of pretreatment disclosed a more pronounced ^{14}C-NA formation (Table I The amount of ^{14}C DA recovered were in both cases approximately the same as that of untreated

Pyridoxal phosphate in a concentration of $1\ 2\times10^{-4}$ *M in the incubation medium* had not any effect on the formation of ^{14}C DA and ^{14}C NA from ^{14}C DOPA These results thus show that there is no lack of the co-enzyme pyridoxal phosphate for DOPA decarboxylase during the experimental conditions used Pretreatment of the animals with 6 OH DA (2×50 mg/kg *i v* 16 hrs interval 2 hrs which causes a disappearance of the endogenous NA and a degeneration of the adrenergic nerves resulted in strongly reduced formation of ^{14}C-NA and ^{14}C DA respectively when incubating the atria in ^{14}C DOPA (Fig 5)

The subcellular distribution of ^{14}C-NA and ^{14}C DA formed from ^{14}C DOPA in atria from untreated or drug pretreated mice was also investigated and the results are presented in Table II

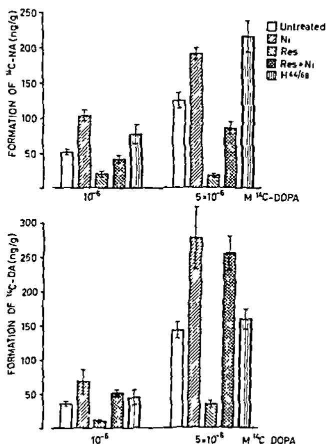


Fig 4 Formation of ^{14}C-NA and ^{14}C-DA in mouse atria from variously pretreated mice The atria had been incubated *in vitro* in 10^{-6} M or 5×10^{-6} M ^{14}C-DOPA for 60 min Each column represents the mean ± S E M of 6—8 determinations

TABLE I Formation of ^{14}C-NA and ^{14}C-DA in atria from mice pretreated with H44/68 (500 mg/kg i p 1 hr or 16 hrs) after incubation in ^{14}C-DOPA (10^{-6} M or 5×10^{-6} M 60 min) *in vitro* Each value represents the mean ± S E M of 4—7 determinations

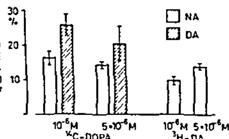
Pretreatment	^{14}C DOPA (M)	^{14}C-NA (ng/g)	^{14}C-DA (ng/g)
Untreated	10^{-6}	51.7 ± 4.1	35.2 ± 3.3
	5×10^{-6}	126.1 ± 11.6	142.7 ± 12.1
H44/68 (1 hr)	10^{-6}	52.2 ± 4.5	33.4 ± 3.3
	5×10^{-6}	147.5 ± 12.9	154.5 ± 17.0
H44/68 16 hrs	10^{-6}	*84.5 ± 11.1*	*42.2 ± 11.4*
	5×10^{-6}	218.9 ± 23.0	159.8 ± 14.2

Formation of ^{3}H-NA from ^{3}H-DA

Atria were incubated for various periods of time in different concentrations of ^{3}H-DA The formation of ^{3}H-NA was fairly constant up to 60 min for all concentrations used (Fig 6) The mean formation of ^{3}H-NA when incubating atria in 10^{-6} M ^{3}H-DA was 166.7 ng/g/hr and in 5×10^{-6} M ^{3}H-DA 246.8 ng/g/hr The effect of pre-

Fig 5 Formation of ^{14}C NA and ^{14}C DA from ^{14}C DOPA and ^{3}H NA from ^{3}H DA in mouse atria from mice pretreated with 6 OH DA (2×50 mg/kg *i v* 16 hrs interval 2 hrs) The atria had been incubated in various concentrations of ^{14}C-DOPA or ^{3}H DA (10^{-6} M or 5×10^{-6} M) for 60

t ons



treatment with reserpine (10 mg/kg *i p* 16 hrs) and/or nialamide (100 mg/kg *i p* 2 hrs) or H44/68 (500 mg/kg *i p* 16 hrs) on the formation of ^{3}H NA from ^{3}H DA is shown in Fig 7 and the subcellular distribution of ^{3}H NA in Table III For comparison the subcellular distribution of ^{3}H NA taken up and accumulated in atria from untreated reserpine+nialamide or H44/68 pretreated mice incubated in 5×10^{-6} M ^{3}H NA for 60 min is shown in Table IV

As in the experiments with ^{14}C DOPA, there was observed a very pronounced reduction in the formation of ^{3}H NA in atria from mice pretreated with 6-OH DA (2×50 mg/kg *i v* 16 hrs interval 2 hrs) (Fig 5)

Fluorescence histochemistry

The histochemical fluorescence method of Falck and Hillarp for the demonstration of biogenic monoamines can visualize NA DA and DOPA The spectral characteristics of the fluorophors (6 7-dihydroxy 3,4 dihydroisoquinolines) formed from these compounds are, however, almost identical and can thus not be differentiated between in the fluorescence microscope (see Jonsson 1967) The relative fluorescence yields from all these compounds are almost the same compared on a molar basis (Jonsson 1967)

TABLE II Subcellular distribution of ^{14}C-NA and ^{14}C-DA in atria of variously pretreated mice The atria had been incubated *in vitro* in 10^{-5} M ^{14}C-DOPA for 60 min Six atria were pooled for each determination Each value represents the mean ± S E M of 8—10 determinations

Pretreatment	^{14}C NA				^{14}C-DA			
	P_1 (ng)	P_2 (ng)	S (ng)	$\frac{P_2}{P_2+S}\times100$	P_1 (ng)	P_2 (ng)	S (ng)	$\frac{P_2}{P_2+S}\times100$
Untreated	0 63 ± 0 07	0 76 ± 0 06	1 66 ± 0 11	31 6 ± 2 7	0 63 ± 0 08	0 57 ± 0 06	2 69 0 12	1 ɔ ± 1 9
Reserpine (10 mg/kg *i p* 16 hrs) + *nialamide* (100 mg/kg *i p* 2 hrs)	0 4ɔ ± 0 03	0 68 ± 0 0	1 41 ± 0 08	32 6 ± 3 8	0 62 ± 0 08	0 6ɔ ± 0 08	ɔ 78 ± 0 13	10 1 ± 1 2
H44/68 (500 mg kg *i p* 16 hrs)	0 90 ± 0 06	1 30 ± 0 11	3 04 ± 0 13	30 0 ± 2 5	0 37 ± 0 06	0 47 ± 0 06	3 12 ± 0 13	13 1 ± 2 1

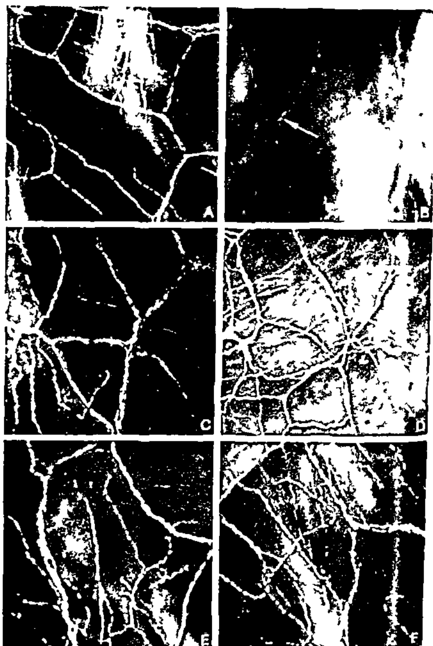


Fig 8 Histochemical demonstration of catecholamines in mouse atria prepared as whole mounts ×250

A *Untreated* The adrenergic nerves form a net work of characteristic varicose nerves. B *H44/68 pretreatment* Only a few weakly fluorescent adrenergic nerves → can be observed. C. *Reserpine + nialamide pretreatment* Incubation in 5×10^{-6} M DA for 30 min Adrenergic nerves with a varicose appearance are seen D *Reserpine + nialamide pretreatment* Incubation in 5×10^{-6} M NA for 30 min The adrenergic nerves disclose a more smooth appearance and prominent non terminal axons → F *H44/68 pretreatment* Incubation in 10^{-5} DA for 30 min. Adrenergic nerves with a varicose appearance can be seen F *H44/68 pretreatment* Incubation in 10^{-5} M DOPA for 60 min Adrenergic nerves with a varicose appearance are seen

nerves but in this case the nerves displayed a more varicose appearance (see Fig 8C) The fluorescence of the nerves was more intense after incubation in DA than in DOPA using the same medium concentrations

Incubation of atria from H44/68 pretreated mice in NA, DA or DOPA (10^{-6} M, 5×10^{-6} or 10^{-5} M, 30 or 60 min) resulted in a restoration of the fluorescence of the adrenergic nerves, which disclosed a morphology just as that of untreated, both regarding the nerve terminals and the non terminal axons (see Fig 8E and 8F) It was necessary to use higher concentrations of DOPA in the medium to obtain the same fluorescence intensity of the nerves as compared with DA and NA It was an often observed phenomenon that after incubation of the tissue in DA the fluorophors formed and/or amines more easily diffused from the nerves during the histochemical reaction

6-OH DA (2×50 mg/kg i v 16 hrs and 2 hrs) produced a complete disappearance of the fluorescent adrenergic nerves Incubation of atria from such 6-OH-DA pretreated mice in DOPA or DA did not restore any nerves Using medium concentrations higher than 10^{-5} M (30 min) resulted only in a gradually increasing evenly distributed background fluorescence

Discussion

The predominating catecholamine in the peripheral adrenergic nerves is considered to be NA In guinea pig and rabbit atria DA has also been found within nerve structures (Angelakos *et al* 1963), but data on the DA content in mouse heart tissue is lacking In the rat heart NA is the only catecholamine found even after DA-β-hydroxylase inhibition by diethyldithiocarbamate (Carlsson *et al* 1966)

The main pathway in the biosynthesis of NA has been shown to be tyrosine→DOPA→DA→NA The different enzymes involved are present in most tissues and organs and are considered to be almost exclusively associated with the presence of cells containing catecholamines above all the adrenergic neurons (for references see Iversen 1967) This latter statement is also corroborated by the findings in the present study since the synthesis of ^{14}C NA and ^{14}C-DA *from* ^{14}C DOPA and also of ^{3}H NA from ^{3}H DA is very markedly reduced after pretreatment with 6-OH-DA A large dose of this drug as used in this study causes very rapidly a severe damage of the uptake storage mechanisms of NA in the adrenergic nerves and later a selective destruction of the nerves (see Jonsson and Sachs 1970) There is, however, a small synthesis of NA and DA in the atria from 6-OH DA pretreated mice and the question arises where this synthesis occurs In all probability it occurs in adrenergic nerve terminals not destroyed or not completely destroyed by the 6-OH-DA treatment The 6 OH DA dose used has been found to cause a reduction of the NA content of mouse heart to about 10 % of untreated indicating that not all nerves are completely destroyed Jonsson and Sachs 1970, see also Haeusler *et al* 1969) The remaining NA content of the tissue however need not necessarily be strictly correlated to the persisting synthetic capacity which for NA was 10—15 % of normal and for DA 20—

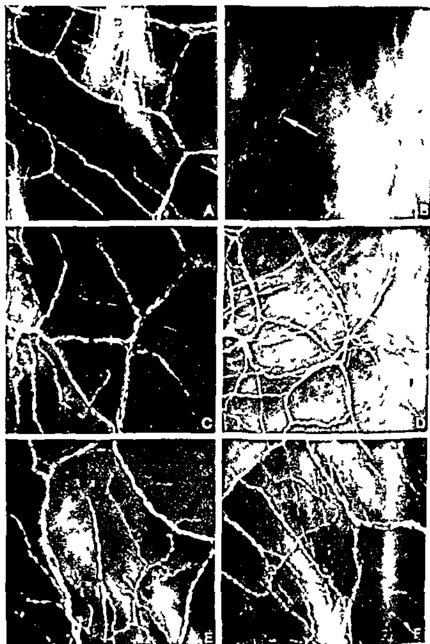


Fig 8 Histochemical demonstration of catecholamines in mouse atria prepared as wh mounts ×250

A *Untreated* The adrenergic nerves form a net work of characteristic varicose nerves *H44/68 pretreatment* Only a few weakly fluorescent adrenergic nerves (→) can be observ C *Reserpine+nialamide pretreatment* Incubation in 5×10^{-6} M DA for 30 min Adrener

that reserpine inhibits formation of NA from DA (Stjarne *et al* 1967) The decrease in the amount of ^{14}C-DA found after incubation of atria from reserpine pretreated mice in ^{14}C DOPA is certainly also a consequence of a blockade of the granular uptake mechanism, since this blockade will cause newly formed DA to be mainly extragranularly localized where it is readily available for degradation by MAO A reduced formation of unchanged DA was also found by Rutledge and Weiner (1967) and Roth and Stone (1968), while the metabolites of DA were increased The inhibition of the NA synthesis by reserpine is thus localized at the DA β hydroxylase step This is also supported by results obtained by Jonason and Rutledge (1969) using α methylated amine analogues

There is, however, not a complete blockade of the NA synthesis from DOPA and DA by reserpine (*cf* Glowinski *et al* 1966) and the blockade can be counteracted by MAO inhibition produced by nialamide In this case the NA formed is certainly localized in the amine storage granules since the proportion of radioactive NA formed from ^{14}C DOPA or ^{3}H DA present in the P_2 fraction, which is considered to contain the amine storage granules (see Jonsson and Sachs 1969) was fairly similar as that of untreated This is also supported by the histochemical results since the adrenergic nerves in these cases displayed a varicose appearance which is a sign of a granular binding of amine (Malmfors 1965, Jonsson and Sachs 1969) The newly formed NA is considered to be incorporated in a special storage pool of the granules readily available for release selectively by nerve impulses (Haggendal and Malmfors 1969, Malmfors 1969, see also Kopin *et al* 1968) This in contrast to the situation when exogenous NA is taken up and accumulated after reserpine+nialamide pretreatment when the accumulated NA is mainly extragranularly distributed as indicated by the subcellular distribution data (see Table IV) and the fluorescence histochemical results (see Fig 8D) showing smooth adrenergic nerves with no distinct varicosities In this case NA is not available for release by nerve stimulation (Malmfors 1969, Farnebo and Hamberger 1970) although there is a small uptake and retention of NA in the granules which is reserpine resistant (Stitzel and Lundborg 1967, Jonsson and Sachs 1969 see also Euler and Lishajko 1963) Reserpine thus seems to be able to more efficiently block the uptake of NA in the granules than to block that of DA but part of the latter compound is converted to NA in the granules The proportion of unchanged DA in the P_2 fraction is however, approximately the same as that of NA exogenously administered after reserpine+nialamide pretreatment The reserpine resistant uptake of NA and DA in the granules thus seems to be similar The lower proportion of DA found in the P fraction compared with NA both in untreated and H44/68 pretreated may be explained by a lower affinity of DA to intact amine storage granules (*cf* Musacchio *et al* 1965)

The depleting action of reserpine on tissue stores of catecholamines is considered to be due to both inhibition of formation and impairment of storage although it cannot at present be stated anything about which is the most important process

The conversion of tyrosine→DOPA is catalyzed by tyrosine hydroxylase
process is considered to be the rate limiting step in the NA biosynthesis (

1965 see also Udenfriend 1966) H44/68 the methylester of α methyl p tyrosine is a potent inhibitor of the tyrosine hydroxylase and injected in a large dose causes a marked depletion of the endogenous NA (see Andén et al 1966 Corrodi and Hanson 1966 Corrodi and Malmfors 1966) This depletion is dependent on the nerve impulse activity of the neuron H44/68 pretreatment (16 hrs) resulted in an increased formation of radioactive NA both from ^{3}H DA and ^{14}C DOPA while the ^{14}C DA formation from ^{14}C DOPA was not significantly increased The subcellular distribution of formed catecholamines was the same as that of untreated and so was the fluorescence morphological picture The observed increase in NA formation after H44/68 is consistent with the results of Spector et al (1965) and is certainly in part due to decreased competition from endogenous DA for the hydroxylation by DA β hydroxylase but may also in part be due to increased storage sites available for the newly synthesized NA After the H44/68 treatment used in this study there is a marked depletion of the endogenous NA to about 40 % of untreated (Sachs 1970) while both the axon membrane pump and the granular uptake mechanism of the neurons operate (Jonsson and Sachs 1969) H44/68 itself with the dose used does not affect the membrane pump and is not an MAO inhibitor (Jonsson et al 1969) The hypothesis of increased number of storage sites is supported by the finding that the NA synthesis was only slightly increased after a short time of H44/68 treatment (1 hr) when more storage sites were occupied by endogenous NA The inhibition of tyrosine hydroxylase is however very efficient already after 1 hr (see Spector et al 1965)

Earlier studies have shown that after H44/68 treatment it is difficult completely to replenish the reduced NA stores by exogenous administration of NA both *in vivo* and *in vitro* (Jonsson et al 1969 Sachs 1970) This may be due to a pool of the amine storage granules that has to be filled by the ordinary synthetic pathway (via the β hydroxylation step) and that this pool is only in a slow equilibration with the storage pool which can be reached by NA administered exogenously It has also been found that in the rat brain catecholamine levels reach normal values after L-DOPA treatment in animals previously depleted of their catecholamine stores by H44/68 (Corrodi et al 1966) Iversen (1963 has also presented evidence that exogenously administered NA does not mix freely with the endogenous NA store

The incomplete replenishment of the endogenous NA store after H44/68 treatment with exogenously administered NA may in part also be due to H44/68 treatment causes losses of granular proteins of importance for storage of NA It has been reported that ATP DA β hydroxylase and certain granular proteins are released together with NA upon nerve stimulation Blaschko et al 1967 Geffen et al 1969) In view of the increased synthesis of NA after H44/68 treatment this possibility seems however to be of minor importance although it cannot be excluded

Acknowledgements

The investigation has been supported by research grants from the Swedish Medical Research Council (B70-14X 2295 03) and by grants from "Stiftelsen Therese och ...

... Corrodi The skilful technical assistance of Mrs Ulla Britt Finnman and Mrs Eva Lindqvist is gratefully acknowledged

References

ANDEN, N-E, H CORRODI, A DAHLSTRÖM, K FUXE and T HÖKFELT, Effects of tyrosine hydroxylase inhibition on the amine levels of the central monoamine neurons *Life Sci* 1966 *5* 561—568

ANGELAKOS, E T, K FUXE and M L TORCHIANA Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts *Acta physiol scand* 1963 *59* 184—192

BELPAIRE, F and P LADURON, Tissue fractionation and catecholamines—I Latency and activation properties of dopamine β hydroxylase in adrenal medulla *Biochem Pharmacol* 1968 *17* 411—421

BLASCHKO H, P HAGEN and A D WELCH Observations on the intracellular granules of the adrenal medulla *J Physiol* (Lond) 1955 *129* 27—49

BLASCHKO H, R S COMLINE F H SCHNEIDER, M SILVER and A D SMITH, Secretion of a chromaffin granule protein, chromogranin from the adrenal gland after splanchnic stimulation *Nature* (Lond) 1967 *215* 58—59

CARLSSON, A and B WALDECK, On the role of the ... hol O methyltransferase in metabolism ...) 1963 *20* 47—55

CARLSSO... the ar... pendent storage mechanism in ... 47—53

CARLSSO... mech... Suppl ... Mg⁺⁺-ATP dependent storage ... *Acta physiol scand* 1963 *59*

CARLSSON, A M LINDQVIST, K FUXE and T HÖKFELT, Histochemical and biochemical effects of diethyldithiocarbamate on tissue catecholamines *J Pharm Pharmac* 1966 *18* 60—62

CORRODI H and L C F HANSON, Central effects of an inhibitor of tyrosine hydroxylation *Psychopharmacol* 1966 *10* 116—125

CORRODI H and G JONSSON The formald... demon... che...

CORR... tr...

COR... pl... 2 ...

EUL... Effect of reserpine on the uptake of catecholamines in isolated nerve storage granules *Int J Neuropharmacol* 1963 *2* 127—134

FARNEBO L O and B HAMBERG... stimulation *J Pharmacol exp* ...

GEFFEN L B B G LIVETT and ... sheep sympathetic neurones a... *204* 58—59

GLOWINSKI J L L IVERSEN and J AXELROD Storage and synthesis of norepinephrine in the reserpine treated brain *J Pharmacol exp Ther* 1966 *151* 385—399

HAEUSLER G W HAEFELY and H THOENEN Chemical sympathectomy of the cat with 6-hydroxydopamine *J Pharmacol exp Ther* 1969 *170* 50—61

HAGGENDAL J and T MALMFORS, The effect of nerve ... mines taken up ... *5* 33—38

H...

I... ues of the rat

I... *J Pharmacol*

I... uptake and storage of noradrenaline in sympathetic nerves Univ Press, Cambridge 1967

1965 see also Udenfriend 1966) H44/68 the methylester of α methyl p-tyrosine is a potent inhibitor of the tyrosine hydroxylase and injected in a large dose causes a marked depletion of the endogenous NA (see Andén *et al* 1966 Corrodi and Hanson 1966 Corrodi and Malmfors 1966) This depletion is dependent on the nerve impulse activity of the neuron H44/68 pretreatment (16 hrs) resulted in an increased formation of radioactive NA both from ³H DA and ¹⁴C-DOPA while the ¹⁴C-DA formation from ¹⁴C DOPA was not significantly increased The subcellular distribution of formed catecholamines was the same as that of untreated and so was the fluorescence morphological picture The observed increase in NA formation after H44/68 is consistent with the results of Spector *et al* (1965) and is certainly in part due to decreased competition from endogenous DA for the hydroxylation by DA β-hydroxylase but may also in part be due to increased storage sites available for the newly synthesized NA After the H44/68 treatment used in this study there is a marked depletion of the endogenous NA to about 40 % of untreated (Sachs 1970) while both the axon membrane pump and the granular uptake mechanism of the neurons operate (Jonsson and Sachs 1969) H44/68 itself with the dose used does not affect the membrane pump and is not an MAO inhibitor (Jonsson *et al* 1969) The hypothesis of increased number of storage sites is supported by the finding that the NA synthesis was only slightly increased after a short time of H44/68 treatment (1 hr) when more storage sites were occupied by endogenous NA The inhibition of tyrosine hydroxylase is however very efficient already after 1 hr (see Spector *et al* 1965)

Earlier studies have shown that after H44/68 treatment it is difficult completely to replenish the reduced NA stores by exogenous administration of NA both *in vivo* and *in vitro* (Jonsson *et al* 1969 Sachs 1970 This may be due to a pool of the amine storage granules that has to be filled by the ordinary synthetic pathway (via the β hydroxylation step and that this pool is only in a slow equilibration with the storage pool which can be reached by NA administered exogenously It has also been found that in the rat brain catecholamine levels reach normal values after L-DOPA treatment in animals previously depleted of their catecholamine stores by H44/68 (Corrodi *et al* 1966 Iversen 1963 has also presented evidence that exogenously administered NA does not mix freely with the endogenous NA store

The incomplete replenishment of the endogenous NA store after H44/68 treatment with exogenously administered NA may in part also be due to H44/68 treatment causes losses of granular proteins of importance for storage of NA It has been reported that ATP DA β hydroxylase and certain granular proteins are released together with NA upon nerve stimulation Blaschko *et al* 1967 Geffen *et al* 1969 In view of the increased synthesis of NA after H44/68 treatment this possibility seems however to be of minor importance although it cannot be excluded

Acta physiol. scand 1970 80 323—344

From the Department of Pharmacology, University of Uppsala Sweden

Effects of Some Adrenergic Neuron Blockers, Related Quaternary Ammonium Compounds and Guanidine Derivatives on Degenerating Adrenergic Nerves in the Conscious Rat

By

DAG LUNDBERG

Received 3 March 1970

Abstract

LUNDBERG, D *Effects of some adrenergic neuron blockers, related quaternary ammonium compounds and guanidine derivatives on degenerating adrenergic nerves in the conscious rat* Acta physiol scand 1970 *80* 323—344

Adrenergic neuron blockers related quarternary ammonium compounds and guanidine derivatives and some other drugs have been tested on a degenerating nerve effector system in the rat periorbital smooth muscle The main attention was paid to their influence on the degeneration contraction The quaternary ammonium compounds structurally related to bretylium and xylocholine with few exceptions distinctly delayed the onset of the degeneration contraction The guanidine derivatives known to be related to bretylium and xylocholine … The simple compound phenyltrimethylammonium … found to possess actions on the degenerating … induced delay was efficiently reduced by des-

The adrenergic neuron blockers bretylium and β TM 10 have been shown to delay the postdenervation decline of endogenous noradrenaline from sympathetically innervated organs (*bretylium* Benmiloud and Euler 1963 Malmfors and Sachs 1965 *β TM 10* Pluchino *et al* 1970) The two drugs also postpone the onset of the transient excitatory effects seen in denervated organs (*bretylium,* Lundberg 1969, Treister and Barany 1970 *β TM 10,* Langer 1966) which are thought to be related to leakage of adrenergic transmitter from the degenerating nerve endings (Sears and Barany 1960 Coats and Emmelin 1962 Langer 1966) Recently the e bretylium on the degeneration contraction of the sympathetically innervat

bital smooth muscle in the rat have been studied (Lundberg 1969, 1970a and b) The drug was found to delay the onset and—under certain conditions—to change the appearance of the degeneration contraction

In order to get information about what kinds of substances possess these effects in common with bretylium some compounds more or less related to bretylium have been tested on the degenerating adrenergic nerve effector system in the rat periorbital muscle

Materials and methods

Experimental animals

Male Sprague Dawley rats weighing about 200 g were used They were kept in normal day light conditions at around 23° C Commercial rat food pellets (No 210 Anticimex Sollentuna Sweden) and tap water were provided ad lib

Surgical procedure

Under ether anesthesia the right superior cervical ganglion was removed (denervation) On the left side the preganglionic trunk was cut (decentralisation)

Drugs

The following drugs were kindly donated by their manufacturers Bretylium (BW 373C57) tosylate bethanidine (BW 467C60) sulfate BW 383C57 tosylate BW 749C57 chloride and BW 172C58 methosulfate (Dr A F Gre[illegible] Beckenham Kent) Xylocholine (TM 10) bromide [illegible] Kline and French Laboratories Welwyn [illegible] sulfate and Ph CC 18B sulfate (Dr K [illegible] [illegible] holm Sweden) Dimethylphenylpiperazinium iodide (DMPP) (Pharmacia Ltd Uppsala Sweden) Guanethidine b sulfate (Ciba Ltd Basel Switzerland) Commercial phenyltrimethylammonium chloride was used after recrystallization The structures of the substances are shown in Table I and II The drugs were dissolved in 0 9 % NaCl immediately before use and injected s c Doses refer to the salts

Measurement of the degeneration contraction

The contraction of the periorbital smooth muscle was estimated in conscious rats by measuring the size of the palpebral aperture at a distance A special apparatus with an image splitting eyepiece was used For details of the method see Lundberg 1969 Usually the apertures of the two eyes were measured every 1 to 1 1/2 hrs during the course of the experiments However in connection with the injections they were measured just before the injection and about 1/2 hr later *The difference in mean size of aperture between the denervated and the decentralized* (control) side at every occasion of measurement was plotted against the time after denervation The difference curve (the degeneration contraction curve) thus obtained was then analysed from the following points of view

The time course Usually the times corresponding to 50 % of maximum effect on the ascending part (T_{50a}) and on the descending part (T_{50d}) of the curves were used as time of start and time of *end of the degeneration contraction respectively In some experiments* however the curves had two humps In comparisons of such curves either between themselves or with single hump curves an ambiguity arises In these cases therefore the times corresponding to a difference between the sides of 1 0 mm on the first ascending limb ($T_{1\,0a}$) and on the last descending limb ($T_{1\,0d}$) of the curves were used instead of T_{50a} and T_{50d} respectively The difference of 1 0 mm is equivalent to about 50 [illegible] of the peak difference of a normal degeneration contraction *Because the experiments sometimes lasted very long* T_{50d} or $T_{1\,0d}$ values were not always obtained In such groups the median or the possible range of the median was calculated instead of the mean

The duration (the width This was measured as the difference between T_{50d} and T_{50a} or between $T_{1\,0d}$ and $T_{1\,0a}$

The height This was the maximum value noted on the denervated side during the degeneration contraction no regard being taken to the decentralized (control) side

The size Besides the duration and the height the area covered by the difference curve is an expression for the size of the degeneration contraction. The area of the first [illegible] hrs after the onset of the contraction was measured. The control and the experimental rats were [illegible] for equal time intervals after the starts of the contractions

Estimation of the sympathomimetic and the ptosis-inducing effect

The increase in palpebral aperture immediately following an injection of a drug has been considered as a sympathomimetic effect With the doses used this effect always reached its maximum within 1 hr after the s.c. injection and lasted only for 2 to 4 hrs. The magnitude of the excitatory effect was estimated by measuring the difference between the peak value [illegible] and the pretreatment value The time needed for 50 % of the peak effect to disappear was used as a measure of duration of the effect. Sometimes the sympathomimetic effect on the denervated side exceeded that on the decentralized side resulting in a peak on the difference curve The total area of this peak was taken as the magnitude of the asymmetric sympathomimetic effect

The ptosis-inducing effect was studied in rats with intact sympathetic innervation and estimated in the corresponding manner

Student's t test was used for analysis of significance

Results

Screening experiments

16 compounds more or less related to bretylium were screened for functional effects on the degenerating nerve-effector system The drugs were usually injected s.c. at 12 hrs after denervation. Their sympathomimetic- and delaying effect and their influence on the time course of the degeneration contraction were recorded. With a few exceptions the substances were tested at two dose levels 4 mg/kg (n = 4) and 20 mg/kg (n = 2) Moreover the ptosis-inducing effect of most drugs (usually at 4 mg/kg) was estimated on the same, still intact, rats about 1 week before the denervation experiment The control animals were given 0.9 % sodium chloride instead of drug The results are shown in Table I and II The bretylium data uppermost in Table I from Lundberg 1970b are put in for comparison It is seen that bretylium given at 12 hrs after denervation, besides delaying the onset of the degeneration contraction increased the duration of the contraction and changed the normal single hump appearance of the contraction into a double hump one The drug also had a tendency to induce an asymmetric sympathomimetic effect i.e. to induce a greater sympathomimetic effect on the denervated than on the decentralized side This was so in 5 out of 12 rats

a *Effects of some quaternary ammonium compounds related to bretylium and xylocholine (TM 10)*

See Table I It is seen that most but not all of the drugs delayed the onset of the degeneration contraction The greatest delay was obtained with phenyltrimethylammonium (PTMA) at 4 mg/kg It was especially long lasting when the contraction was of single hump type The clear-cut difference in delaying activity between p TM 10 and the structurally very close relative TM 10 is notable In 3 out of the 7 rats given 4 mg/kg of PTMA the contraction had the double hump [illegible]

TABLE 1 [illegible]

as 0 if the maximum change in palpebral aperture was less than 0 5 mm The time inter[illegible] tion of the action Values are individual values with their means or means ± S E M The [illegible] only if the treated group contained at least 4 rats Differences between treated groups [illegible]

Drug	Structure	Dose mg/kg	n	Time of start $T_{0.5}$ or $T_{1.0}$ hrs[1]
Bretylium	$C_6H_4(Br)-CH_2-N^+(CH_3)_2(C_2H_5)$	4	12	19 42 ± 0 35 (19 82 ± 0 38)
BW 383C57	$C_6H_4(Br)-CH_2-N^+(CH_3)_3$	4	4	18 17 ± 0 31
		20	2	18 69, 18 38 } 18 53
BTMA (benzyltrimethylammonium)	$C_6H_5-CH_2-N^+(CH_3)_3$	4	4	19 00 ± 0 31
		20[2]	2	—
PTMA (phenyltrimethyl ammonium)	$C_6H_5-N^+(CH_3)_3$	1	2	17 91, 17 75 } 17 83
		4	4	25 53 ± 0 69
		4	3	20 14 (20 70), 19 07 (19 30), 23 92 (24 00) } 21 04 (21 33)
BW 749C57	$C_6H_4(Cl)-CH_2-N^+(CH_3)(CH_2CH_2OH)_2$	4	4	16 62 ± 0 56
		20	2	15 68, 16 46 } 16 07
Xylocholine (TM 10)	$C_6H_3(CH_3)_2-O-CH_2-CH_2-N^+(CH_3)_3$	4	4	17 70 ± 0 51
		20	2	18 96, 18 86 } 18 91
Beta-TM 10	$C_6H_3(CH_3)_2-O-CH(CH_3)-CH_2-N(CH_3)_3$	4	5	14 90 ± 0 14

bretylium or xylocholine (TM 10) and their sympathomimetic effect when given 12 hrs after

Delay of start[1] hrs	Duration $T_{50d}-T_{50a}$ or $T_{1d}-T_{1a}$ hrs	Height (peak aperture on denervated side) mm	Ptosis inducing effect: maximum effect mm	Ptosis inducing effect: duration hrs	Sympathomimetic effect: maximum effect mm	Sympathomimetic effect: duration hrs
	D				0 52 ± 0 16 (den side)	1 3 ± 0 2 (den side)
4 23*** (4 39)	10 37 ± 0 72 (10 32 ± 0 62)	3 95 ± 0 13	1 42 ± 0 29 n = 4	2 7 ± 0 2 n = 4	0 (dec side)	0 (dec side)
2 98***	7 58 ± 0 17	4 34 ± 0 19	0	0	0	0
3 34	8 70, 9 86 } 9 28	4 70, 4 70 } 4 70	n t	n t	1 0, 0 9 } 0 95 (den = dec)	1 8, 2 0 } 1 9 (den = dec)
3 81***	6 58* ± 0 49	4 33 ± 0 038	0	0	1 59 ± 0 11 (den = dec)	1 5 ± 0 3 (den = dec)
—	—	—	—	—	—	—
2 64	>8 94, 8 50	4 88, 5 20 } 5 04	n t	n t	0	0
10 34***	>4 45*	4 08 ± 0 19	0	0	0	0
5 85 (5 90)	>10 26 D (>9 70) >11 23 (11 00) >7 67 (7 80)	4 80, 4 10, 4 20 } 4 37	0	0	0	0
1 43*	6 36 ± 1 08	4 13 ± 0 11	0	0	0	0
0 88	6 56, 5 59 } 6 07	4 70, 4 80 } 4 75	n t	n t	0	0
2 51***	6 81 ± 0 34	4 73 ± 0 048	1 10 ± 0 18	>7 0*	0	0
3 72	7 28, 7 11 } 7 20	4 95, 4 73 } 4 84	n.t	n t	0	0
−0 29	7 19 ± 0 61	4 45 ± 0 092	0	0	0	0

Table I Cont

Drug	Structure	Dose mg/kg	n	Time of start $T_{50\%}$ or $T_{1.0\%}$ hrs[2]
BW 172C58	$C_6H_5{-}CO{-}C_6H_2(CH_3)_2{-}O{-}CH_2{-}CH_2{-}N^+(CH_3)_3$	4	4	16.94 ± 0.13
		20	2	17.66, 15.84 } 16.75
Hemicholinium	$(HO{-}CH_2{-}CH_2{-}N^+(CH_3)_2{-}CH_2{-}CO{-}C_6H_4)_2$	0.1	4	16.11 ± 0.30
		0.5[3]	2	—
Controls (saline)		2 ml/kg	31	15.19 ± 0.18 (15.43 ± 0.20)

[1] $T_{50\%}$ exp — $T_{50\%}$ contr or $T_{1.0\%}$ exp — $T_{1.0\%}$ contr [2] hrs after denervation [3] lethal dose [4] median value

None of the other agents, except bretylium produced this phenomenon TM 10 produced ptosis lasting for more than 7 hrs It was the only drug besides bretylium with measurable such activity BW 383C57 at 20 mg/kg and especially benzyl trimethylammonium (BTMA) at 4 mg/kg showed sympathomimetic activity which was symmetric The other drugs except bretylium lacked excitatory effect

In some treated groups the duration of the degeneration contraction differed significantly from that of the control group However, the present experiment does not allow assessment of the causes of such a change

BTMA at 20 mg/kg and 0.5 mg/kg of hemicholinium proved to be lethal within 5 to 10 min apparently because of respiratory distress With the other drugs there were no toxic symptoms

b *Effects of some guanidine derivatives hexamethonium, mecamylamine and dimethylphenylpiperazine (DMPP)*

See Table II None of the drugs induced any clear-cut delay of the contraction However, there was a slight delay with Ph 881/7 and with hexamethonium Ph CC 18B at 20 mg/kg instead of delaying seemed to start the contraction somewhat prematurely Durations were apparently changed in some groups but there were so few animals in some of these that significance is doubtful There were no double humps No degeneration contraction developed in 2 out of the 5 rats treated with guanethidine In 3 of the 5 rats it was normal The reasons are not clear Bethanidine and especially Ph 881/7 induced ptosis presumably by neuron blocking action, whereas all other guanidine derivatives and DMPP lacked this effect at the doses used

Delay of start[c] hrs	Duration T_{50d}—T_{50a} or $T_{1 ed}$—$T_{1 ea}$ hrs	Height (peak aperture on denervated side) mm	Ptosis-inducing effect		Sympathomimetic effect	
			maximum effect mm	duration hrs	maximum effect mm	duration hrs
1 75*	9 67* ± 0 42	4 78 ± 0 058	0	0	0	0
1 56	11 37 / 10 58 } 10 97	5 17 / 4 83 } 5 00	n t	n t	0	0
0 92	7 37 ± 0 22	3 86 ± 0 24	0	0	0	0
—	—	—	—	—	—	—
—	8 09 ± 0 24 (7 21 ± 0 33)	4 23 ± 0 078	n t	n t	0	0

D = the degeneration contraction curves are of the double hump type
n t — not tested

Mecamylamine (4 mg/kg) and hexamethonium (15 mg/kg) which are established ganglion blockers also induced ptosis but this effect is most probably due to a ganglion block rather than to a neuron block Bethanidine was the only drug which showed a sympathomimetic effect and this was symmetric There was no visible toxic symptom in any treated rat

Delaying effect of different doses of phenyltrimethylammonium (PTMA) or bethanidine

The screening experiment showed that PTMA at 4 mg/kg had a strong delaying effect whereas the same dose of bethanidine lacked such an effect Bethanidine differs structurally from PTMA mainly by having a guanidine group instead of a trimethylammonium group, see Table I and II Both these strong bases are present as cations at physiological pH It seemed to be of interest to test the drugs at some more dose levels Different doses of PTMA or bethanidine were given s c to groups of 5 rats at 11 hrs after denervation The results are illustrated in Fig 1 which also shows the dose-response curve of bretylium published earlier (Lundberg 1970a) The dose-response curve of PTMA is less steep than that of bretylium PTMA at 0 08 mg/kg had almost no effect and the maximum delay obtained was reached at 4 to 7 5 mg/kg In 1 out of the 3 rats treated with 7 5 mg/kg and in the rat given 15 mg/kg of PTMA the degeneration contraction was of the double hump type The rats injected with 7 5 or 15 mg/kg of PTMA suffered seriously from respiratory distress for about 1 hr after the injection. Thus PTMA seems to be much more toxic than bretylium which can be administered s c to rats at 50 mg/kg every 4 hrs for at least 24 hrs (Benmiloud and Euler 1963) Bethanidine was entirely ineffective

TABLE II. The influence on the degeneration contraction of some guanidine derivatives, hexame- effect when given at 12 hrs, if not otherwise stated, after denervation The table also was still intact

Drug	Structure	Dose mg/kg	n	Time of start $T_{60\%}$ or hrs[a]
Guanethidine at 10 hrs[a]	(ring) N CH_2 CH_2 NH C(=NH)NH_2	25	3/5[a]	16 15, 15 40, 14 00 } 15 18
Bethanidine at 11 hrs[a]	(phenyl) CH_2·NH C(=NCH_3)$NHCH_3$	4	5	15 23±0 48
Ph CC 18B	(phenyl) CH_2 CH(CH_3) N(CH_3) $(CH_2)_2$ NH C(=NH)NH_2	4	4	15 46±0 50
		20	2	13 81, 13 76 } 13 78
Ph CC 10C	(phenyl) $(CH_2)_3$ N(CH_3) $(CH_2)_2$ NH C(=NH)NH_2	4	4	14 62±0 20
		20	2	17 53, 14 44 } 15 99
Ph 881/7	(benzo-ring) N CH_2 CH_2 NH C(=NH)NH_2	4	4	15 87±0 64
		20	2	17 70, 16 71 } 17 20
Hexamethonium	CH_3 $\overset{+}{N}$(CH_3)$_2$ CH_2 $(CH_2)_4$ CH_2 $\overset{+}{N}$(CH_3)$_2$ CH_3	4	4	15 33±0 38
		15	2	15 70, 17.50 } 16 60
Mecamylamine	(bicyclic) CH_3, CH_3, CH_3, $NHCH_3$, CH_3	4	4	15 75±0 46
		20	2	15 52, 15 22 } 15 37

thonium, mecamylamine and dimethylphenylpiperazine (DMPP) and their sympathomimetic shows the ptosis inducing effect of the drugs usually injected to the same rats while the innervation

Delay of start[1] hrs	Duration $T_{sed} - T_{sea}$ hrs	Height (peak aperture on denervated side) mm	Ptosis inducing effect		Sympathomimetic effect	
			maximum effect mm	duration hrs	maximum effect mm	duration hrs
—0 01	5 35 7 40 } 7 61 10 10	4 50 4 43 } 4 50 4 57	n t	n t	n t	n t
0 04	7 21±0 75	4 68±0 11	1 50±0 39	2 4±0 7	0 84±0 16 (den = dec)	1 9±0 4 (den = dec)
0 27	6 06*±1 11	3 77±0 23	0	0	0	0
—1 41	6 45 7 39 } 6 92	4 57 4 23 } 4 40	n t	n t	0	0
—0 57	7 37±1 99	3 84±0 26	0	0	0	0
0 80	7 53 10 74 } 9 13	3 90 4 54 } 4 22	n t	n t	0	0
0 68	7 34±0 58	4 26±0 069	2 20±0 18	<7[4]	0	0
2 01	9 00 10 45 } 9 73	4 76 4 75 } 4 76	n t	n t	0	0
0 14	7 97±1 21	3 96±0 20	0 n=8	0 n=8	0	0
1 41	9 20 8 20 } 8 70	4 50 4 55 } 4 53	0 93±0 19 n=5	1 6±0 2 n=5	0	0
0 56	7 97±1 01	4 31±0 19	2 60±0 18	3 6±0 4	0	0
0 18	7 78 8 89 } 8 33	4 60 5 23 } 4 92	n t	n t	0	0

Table II Cont

Drug	Structure	Dose mg/kg	n	Time of start T_{50a} or hrs[2]
DMPP	N N⁺ CH_3 CH_3	1	2	15 93} 15 36} 15 64
Controls (saline)		2 ml/kg	31	15 19 ± 0 18

[1] T_{50a} exp — T_{50a} contr or $T_{1 sa}$ exp — $T_{1 sa}$ contr [2] hrs after denervation [3] in 2 out of 5 rats there were no degeneration contractions [4] median value

n t = not tested

as a delayer, except perhaps at 30 mg/kg (however, $p > 0 05$) This is in agreement with earlier findings that a single injection of 50 mg/kg, but not 25 mg/kg of bethanidine given at 10 hrs after denervation somewhat delayed the start of th degeneration contraction in the rat (Lundberg 1969)

Effects of phenyltrimethylammonium (PTMA) given at different time intervals after denervation

Rats, usually in groups of 5, were treated with single s c doses of PTMA (4 mg/kg) at different time intervals between 6 and 14 hrs after denervation The control animals were untreated The results are shown in Table III and Fig 2 For comparison the results of quite similar experiments with bretylium published earlier (Lundberg 1970b) are also shown in Fig 2 It is seen that the time of injection influenced the effects on the degenerating nerve-effector system similarly but not identically in the PTMA and the bretylium experiments Thus, the delaying effect

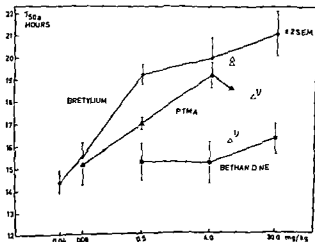


Fig 1 The delaying effect of different doses of bretylium phenytrimethylammonium (PTMA) or bethanidine injected at 11 hrs after denervation The drugs were given to rats in groups of 5 except PTMA at 15 mg/kg and 7 5 mg/kg which was injected only to 1 and 3 rats respectively The individual values of these rats are shown as open triangles T_{50a} is the time of half maximum development of the degeneration contraction The bretylium curve is from Lundberg 1970a

[1] contraction curve is of double hump type

Delay of start[1] hrs	Duration $T_{60}—T_{90}$ hrs	Height (peak aperture on denervated side) mm	Ptosis-inducing effect maximum effect mm	Ptosis-inducing effect duration hrs	Sympathomimetic effect maximum effect mm	Sympathomimetic effect duration hrs
0 45	8 45 / 10 62 } 9 54	4 75 / 4 80 } 4 78	n t	n t	0	0
—	8 09 ± 0 24	4 23 ± 0 078	n t	n t	0	0

of both PTMA and bretylium was distinctly increased when the injection was given close to the expected onset of the degeneration contraction *i e* at 12 to 14 hrs after denervation Also when given late, PTMA like bretylium produced an asymmetric sympathomimetic effect and a tendency to double hump contractions The excitatory effect did not in any case last for more than 5 hrs The two drugs however, also differed in some respects Bretylium as distinguished from PTMA significantly delayed the contraction even when given at 6 hrs after denervation The maximum delay obtained by bretylium was significantly longer than that of PTMA ($p < 0.001$) The big step in the increase in delaying effect was achieved at earlier injections with PTMA than with bretylium Furthermore the asymmetric sympathomimetic action was more pronounced after the PTMA than after the bretylium injections

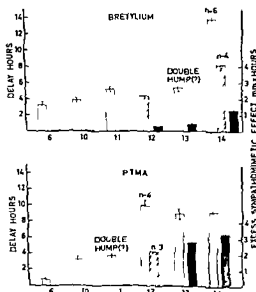


Fig 2 The delaying and the sympathomimetic effects of 4 mg/kg of bretylium or phenyltrimethylammonium (PTMA) injected at different time intervals after denervation The delaying effect shown as open or hatched bars is calculated as the difference between the mean T_{50} (the time of half maximum development of *the degeneration contraction*) of a treated group and that of the control group An open bar represents the delay of a group with single hump contraction curves and a hatched bar that of a group with double hump curves The excess sympathomimetic effect shown as filled bars is the effect on the denervated side minus that on the decentralized control side It is measured as the area covered by the sympathomimetic peak of the difference curve see Methods Means ± S E M are shown
The bretylium groups (from Lundberg 1970b) had n = 10 except in the 6 hrs (n = 5) and the 12 hrs (n 12 groups The PTMA groups had n = 5 except at 12 hrs where there were 7 animals

TABLE III The influence on the degeneration contraction of 4 mg/kg of phenyltrimethylammon um There were two kinds of contraction curves single hump and double hump In see also legend to Table I The maximum effect of the sympathomimetic action was val after the injection needed for 50 % of the maximum effect to disappear was taken as the area covered by the sympathomimetic peak on the difference curve The values
The statistical significance of the difference between a treated group and the control groups were not tested * = p < 0 05 ** = p < 0 01 and *** = p < 0 001

Degeneration contraction

Time of injection hrs[2]	n	Time of start T_{sos} or $T_{1\,os}$ hrs[2]	Delay of start[1] hrs	Duration $T_{sod}-T_{sos}$ or $T_{1\,od}-T_{1\,os}$ hrs	Height (peak aperture on denervated side) mm
6	5	16 21 ± 0 25	0 73	9 76* ± 0 56	4 63 ± 0 096
10	5	18 78 ± 0 35	3 30***	6 49 ± 0 45	4 07 ± 0 10
11	5	19 24 ± 0 30 (19 68 ± 0 55)	3 76*** (3 99)	>10 97[3] D (10 10—11 60)[3]	4 64 ± 0 10
12	3	(20 70) (19 30) (21 33) (24 00)	(5 64)	(>9 70) D (11 00) (7 80)	4 80 4 10 4 37 4 20
	4	25 53 ± 0 69	10 05***	>4 45[3]	4 08 ± 0 19
13	5	24 48 ± 0 60	9 00***	6 48 ± 0 71	3 81 ± 0 17
14	5	24 72 ± 0 55	9 24***	5 90* ± 0 74	4 02 ± 0 13
Controls	15	15 48 ± 0 34 (15 69 ± 0 36)	—	7 80 ± 0 35 (7 23 0 48)	4 24 ± 0 13

[1] T_{sos} exp — T_{sos} contr or $T_{1\,os}$ exp — $T_{1\,os}$ contr
[2] hrs after denervation
[3] median or range of the median value

Interaction between phenyltrimethylammonium (PTMA) and desmethylimipramine (DMI) and lack of interaction between PTMA (or bretylium) and anticholinergic drugs

It has previously been shown that the bretylium induced delay is reduced by inhibitors of the axonal amine pump such as DMI (Lundberg 1970a) It seemed worthwhile to test the effect of DMI also on the PTMA induced delay Besides DMI some anticholinergic drugs were included in the experiment and tested as inhibitors because of the cholinergic look of PTMA and bretylium and their known cholinergic activities (for references see Discussion) The schedule of treatment and the results are shown in Table IV and V All drugs were given s c Table IV shows

(PTMA) and its sympathomimetic effect when given [illegible]

group was tested only if the treated group contained at least 4 rats. Differences between treated

Sympathomimetic effect		
Maximum effect mm	Duration hrs	Excess effect on denervated side mm × hrs
n t	n t	n t
0	0	0
0	0	0
0	0	0
0	0	0
1 37±0 16 (den side) 0 (dec side)	1 6±0 09 (den.side) 0 (dec.side)	2 70±0 81
1 10±0 18 (den.side) 0 (dec.side)	2 0±0 3 (den.side) 0 (dec.side)	3 18±0 96
0	0	0

D = the degeneration contraction curves are of the double hump type
n t = not tested.

results from experiments in which the tested inhibitor was given after PTMA or bretylium The bretylium and the bretylium DMI groups are from Lundberg 1970a It is seen that DMI but not methylatropine significantly reduced the delay induced by PTMA Nor was the bretylium induced delay reduced by hexamethonium. From a comparison of the PTMA DMI experiment with the similarly designed bretylium DMI experiment it is evident that PTMA is much more susceptible than bretylium to counteraction by DMI The durations of the degeneration contraction in the PTMA and the PTMA methylatropine groups were short. This effect probably was related to the strong sympathomimetic effect of the [illegible] of PTMA see Table III during which the degenerating nerve

TABLE IV. [illegible]

Schedule of treatment	n	Time of start T_{50a} hrs[1]	Reduction of delay (D) %	Duration $T_{50d}-T_{50a}$ hrs	Height (peak aperture on denervated side) mm
PTMA (4 mg/kg) at 13 hrs[1]	5	24 48 ± 0 60	—	6 48 ± 0 71	3 81 ± 0 17
		p<0 001		p<0 05	p<0 01
+ DMI (10 mg/kg) at 15 hrs	5	15 98 ± 0 32	92	10 06 ± 1 28	4 59 ± 0 11
PTMA (4 mg/kg) at 13 hrs	5	24 48 ± 0 60	—	6 48 ± 0 71	3 81 ± 0 17
		n s		n s	n s
+ methylatropine (4 mg/kg) at 15 hrs	5	23 98 ± 0 39	5 4	4 90 ± 0 60	4 18 ± 0 22
Bretylium (4 mg/kg) at 13 hrs	10	20 51 ± 0 33	—	10 06—12 72[2]	4 75 ± 0 11
		p<0 001		n t	p<0 05
+ DMI (10 mg/kg) at 15 hrs	10	18 35 ± 0 24	41	7 71 ± 0 34	5 14 ± 0 088
Bretylium (4 mg/kg) at 10 hrs	10	18 98 ± 0 27	—	9 05—9 78[2]	4 94 ± 0 090
		n s		n t	n s
+ *hexamethonium (15 mg/kg) at 12 hrs*	5	*19 72 ± 0 62*	*—20*	*7 46 ± 0 46*	*4 90 ± 0 093*
Methylatropine (4 mg kg) alone at 15 hrs	5	15 19 ± 0 19	—	6 16 ± 0 82	3 82 ± 0 13
		n s		p<0 05	n s
Saline (2 ml/kg) alone at 12 hrs	10	15 22 ± 0 25	—	8 29 ± 0 33	4 11 ± 0 10

[1] time of start and time of injection are expressed in hrs after denervation
[2] range of the median value

n s = not statistically significant
n t = significance of difference not tested

quite a lot of transmitter In the PTMA-DMI group the excitatory effects of the PTMA-injection had not yet stopped when the degeneration contraction started Thus, to a certain extent, the sympathomimetic effect contributed to the peak of the degeneration contraction This could explain the difference in duration and height of the contraction between the PTMA- and the PTMA-DMI groups The ability of DMI to increase the height of the contraction as in the bretylium-DMI- and the PTMA-DMI experiments, has been discussed earlier (Lundberg 1970a)

From Table V it is evident that treatment with 4 mg/kg of atropine or mecamylamine given at 13 5 hrs after denervation did not significantly reduce neither the

TABLE V. The effect of pretreatment with atropine or mecamylamine on the phenyltrimethylammonium (PTMA) induced delay of the degeneration contraction and on the sympathomimetic effect of PTMA. [illegible]

Schedule of treatment	n	Time of start T_{sta} hrs[1]	Reduction of delay (D) %	Duration $T_{end}-T_{sta}$ hrs	Height (peak aperture on denervated side) mm	Excess sympathomimetic effect mm × hrs
PTMA (4 mg/kg) at 14 hrs[1]	5	24 72±0 55	—	5 90±0 74	4 02±0 13	3 18±0 96
		n s		n t	$p < 0.05$	n.s
Atropine (4 mg/kg) at 13 5 hrs + PTMA (4 mg/kg) at 14 0 hrs	5	23 80±0 21	9 7	5 46 (median)	3 49±0 11	3 55±0 75
PTMA (4 mg/kg) at 14 hrs	5	24 72±0 55	—	5 90±0 74	4 02±0 13	3 18±0 96
		n s		n s	n.s	n s
Mecamylamine (4 mg/kg) at 13 5 hrs + PTMA (4 mg/kg) at 14 0 hrs	5	23 75±0 23	10	5.37±0 39	3 72±0 15	2 58±0 74
Atropine (4 mg/kg) alone at 13 5 hrs	5	15 05±0 34	—	6 31±1 15	3 98±0 20	—
		n s		$p < 0.05$	n s	
Saline (2 ml/kg) alone at 12 hrs	10	15 22±0 25	—	8 29±0 33	4 11±0 10	—

[1] time of start and time of injection are expressed in hrs after denervation

delay nor the asymmetric sympathomimetic effect induced by 4 mg/kg PTMA given 1/2 hr later Methylatropine (4 mg/kg) injected alone at 15 hrs see Table IV, or the similar treatment with atropine at 13 5 hrs after denervation did not change the time of onset of the degeneration contraction However, both drugs decreased the duration and (to a less degree) the height of the contraction. This tendency is also seen in Table VI which shows the influence of some antinicotinic and antimuscarinic drugs on the size of the degeneration contraction measured as integrated area of the contraction curve The antinicotinics mecamylamine and hexamethonium did not change the size while the antimuscarinics atropine perhaps and methylatropine certainly were effective According to Ehinger (1966) the smooth muscle of the rat eye lid has a sparse cholinergic innervation

Lack of sympathomimetic action by bretylium or phenyltrimethylammonium (PTMA) given after the degeneration contraction

Simultaneously with the development of the degeneration depletion of noradrenaline from adrenergic nerve endings the function of the axonal amine pump deteriorates (Malmfors and Sachs 1965 Smith *et al* 1966) The excitatory effect of bretyli

TABLE VI The effect of treatment with antinicotinic or antimuscarinic drugs on the size of the degeneration contraction The contractions started after the normal interval T_{100} was 15 05—15 75 hrs after denervation Comparisons are between a treated group and its corresponding control group

Schedule of treatment	n	Area covered by curve mm × hrs
Mecamylamine (4 mg/kg) at 12 hrs[1]	4	12 28±1 84
Hexamethonium (4 mg/kg) at 12 hrs	4	12 09±2 11
NaCl at 12 hrs	10	12 39±1 01
Atropine (4 mg kg) at 13 5 hrs	5	10 90±1 72
Methylatropine (4 mg/kg) at 15 hrs	5	8 05±0 86 p < 0 001
NaCl at 13 5 hrs	10	13 45±0 70

[1] *time of injection is expressed in hrs after denervation*

or PTMA given around the start of the degeneration contraction is stronger on the denervated than on the decentralized side (Lundberg 1970b and this paper) This asymmetric effect could be due to diminished reuptake on the denervated size of the transmitter released by the drugs The source of the released amine could be either the adrenals or the degenerating nerve terminals The site of the releasing action of the two drugs were tested as follows The two groups of rats treated with bretylium at 6 hrs and PTMA at 10 hrs respectively see Fig 2 and Table III were given another dose (4 mg/kg) of the respective drug at about 30 hrs after denervation Then the degeneration contraction were finished in all rats but in 1 of each group and by all probability most of the transmitter had left the nerve endings The two animals with a slight contraction still going on at the time of injection were the only ones which had measurable excitatory effects This finding indicates that the sympathomimetic effect on degenerating nerves induced by bretylium or PTMA is due only to actions on the nerve endings of the effector organ

Lack of delaying effect of bethanidine or hexamethonium even when injected shortly after the start of the degeneration contraction

The delaying effect of bretylium or PTMA is very much increased in injections close to the expected start of the degeneration contraction see Fig 2 Thus after reaching a certain stage of degeneration the nerve endings seem to become more sensitive to the delaying substances It is also known that bretylium injected during an ongoing degeneration contraction interrupts the contraction and displaces the latter part for several hrs (Lundberg 1970b The lack of delaying effect of e g bethanidine or hexamethonium when given at 11 to 12 hrs after denervation see Fig 1 and Table II, could be due to a too early injection To exclude this possibility rats were treated with bethanidine (4 mg/kg and hexamethonium (15 mg/kg) respectively at 15

TABLE VII The effect of bethanidine or hexamethonium on the course and size of the degeneration contraction when injected shortly after the start of the contraction T_{50a} and T_{50d} are times of half maximum effect on the ascending and descending phase of the contraction, respectively Comparisons are between a treated group and the control group There were no statistically significant differences

Schedule of treatment	Time of start T_{50a} hrs[1]	Duration T_{50d}—T_{50a} hrs	Height (peak aperture on denervated side) mm	Area covered by curve mm × hrs
Bethanidine (4 mg/kg) at 15 0—15.5 hrs[1] n 5	15 16±0 10	9 07±1 81	4 92±0 70	14 75±2 49
Hexamethonium (15 mg/kg) at 15 5—17 0 hrs[1] n = 4	15 62±0 87	7 33±1 10	4 06±0 23	12 03±2 62
Controls n = 5	15 32±0 35	7 78±0 33	4.24±0 14	13 63±0 88

[1] time of start and time of injection are expressed in hrs after denervation

to 17 hrs after denervation when the contractions had just started The results are shown in Table VII The time course and the size of the contraction were not significantly changed by any of the drugs

Discussion

Bretylium has been found to delay the onset of the degeneration contraction of a sympathetically innervated smooth muscle in the rat and under certain conditions to change the shape of the contraction Moreover the initial excitatory effect of bretylium *i e* the sympathomimetic effect was increased at injections given at 12 to 14 hrs after denervation which is the time interval just preceding the onset of the normal degeneration contraction (Lundberg 1969, 1970b) Bretylium has a complex pharmacology with several different actions mainly located to postganglionic sympathetic nerves (Boura and Green review 1965) The mechanism of the delaying action is not known However it does not appear to be related to the neuron blocking or the monoamine oxidase inhibiting activity of the drug (Lundberg 1970b and c The aim of the present study was mainly to get an impression of firstly what kinds of substances share with bretylium the presented actions on degenerating adrenergic nerves and secondly if there seems to be any correlation between the delaying action and other previously known actions of delaying drugs

The present screening experiment showed that not only bretylium but also quaternary ammonium compounds related to bretylium and xylocholine (TM 10) and the simple compounds benzyltrimethylammonium (BTMA) and phenyltrimethylammonium (PTMA) with few exceptions had clear-cut delaying effects However

the guanidine derivatives which otherwise are closely related to bretylium pharmacologically, *seemed to lack such an effect* This difference in action between the quaternary ammonium compounds and the guanidine derivatives is strikingly illustrated by a comparison of BTMA with bethanidine see Table I and II Structurally the two compounds differ mainly at the basic head of the molecule BTMA having a quaternary ammonium group and bethanidine a guanidine group Both are present as cations at physiological pH The intramolecular distance between the centre of the basic group and the ring of the two compounds differ being longer in bethanidine than in BTMA However this distance in the bethanidine molecule in exended configuration is not larger than that of xylocholine which is an efficient delayer Thus the difference in delaying action between BTMA and bethanidine *might be due to the different basic groups rather than to a difference in length of* the molecules Apart from being more toxic and more sensitive to the inhibitory action of desmethylimipramine the simple compound PTMA had actions on the degenerating nerve effector system very similar to those of bretylium Thus the main structural features essential for a delaying drug are found in the structure of PTMA It is easily seen that neither the bromine on the ring the ethyldimethylammonium group nor the methylene linkage in bretylium are essential for the delaying effect

The selective accumulation and retention of isotopically labelled bethanidine in the intact adrenergic neurons of the cat resembles that of bretylium (Boura Duncombe Robson and McCoubrey 1962) This fact and the present finding that *bethanidine did not delay even when injected just after the start of the degeneration* contraction—when the sensitivity of the degenerating nerve to bretylium is very prominent (Lundberg 1970b)—indicates that the lack of delaying effect of bethanidine is not due to inexpedient pharmacokinetic factors but to low efficiency at the critical delaying sites in the adrenergic neurons

The Boura group (1960 and 1961) has studied three of the delaying drugs shown in Table I under comparable conditions with respect to (i) the capacity of selective accumulation and persistence of the isotopically labelled drug in intact postganglionic adrenergic nerves (ii) the adrenergic neuron blocking activity *in vivo* and (iii) intrinsic neuron blocking activity tested on the stimulated isolated rabbit ileum (Finkelman preparation) The respective orders of potency of the drugs at the adrenergic nerve endings are roughly as follows

Delaying activity rat see Table I	*bretylium* > BW383C57 > BW172C58
Initial accumulation (at 2—3 hrs after inj) cat	bretylium > BW383C57 > BW172C58
Persistence of drug (at 12—18 hrs after inj) cat	bretylium > BW172C58 > BW383C57
Persistence of neuron block in vivo cat	bretylium > BW172C58 > BW383C57
Intrinsic neuron blocking activity	BW172C58 > bretylium > BW383C57

It is seen that the delaying action seems rather to be related to the capacity of initial accumulation of the drug than to any other parameter presented Thus the

difference in delaying activity between the drugs could be due to different affinity for binding sites in the neurons This putative importance of the capacity of accumulation in the nerve endings is in accordance with an earlier proposal (Lundberg 1970a) that the start of a degeneration contraction delayed by bretylium is due to sufficiently low level of the drug at the critical sites and thus is related to the amount of the drug initially taken up and to the rate of its disappearance from the degenerating nerve endings It is also evident that the delaying effect does not seem to be linked to the neuron blocking action This view, which has been presented earlier (Lundberg 1969 and 1970b) is also favoured by the following In the present experiments only xylocholine (TM 10) and bretylium showed ptosis inducing effect in rats with intact adrenergic nerves at clearly delaying doses This effect is by all probability due to adrenergic neuron block The other delaying drugs did not show any ptosis inducing activity The guanidine derivatives bethanidine and Ph 881/7 very clearly induced ptosis but nevertheless did not delay Furthermore, it has been reported (Long Wong and Witt 1965) that BTMA which seems to be a good delayer see Table I did not inhibit nerve stimulatory effects on isolated rabbit ileum even at very high concentrations However, the adrenergic neurons seem to be more sensitive to the delaying drugs after a certain stage of degeneration has been entered see Fig 2 Since this may indicate that degenerating neurons treat the drugs differently it appears difficult to draw definite conclusions from comparisons of results obtained with intact and with degenerating nerves

Out of the 7 substances with delaying effect only bretylium BW 383C57 and BTMA had measurable sympathomimetic effect when given at 12 hrs after denervation Moreover, bethanidine showed sympathomimetic activity without delaying Thus there seems to be a dissociation also between the sympathomimetic and the delaying actions of the drugs

At detailed studies all the drugs tested above for delaying activity have shown some kinds of action at cholinergic sites BTMA and PTMA have both muscarinic and nicotinic properties *in vivo* even at doses lower than 0 1 mg/kg (*cat* Hunt 1926 *dog* Alles 1944) The latter author found that the muscarine like activity of BTMA and PTMA was 40 and 5 times that of tetramethylammonium respectively The nicotine like activity of the drugs was less prominent being 1/4—1/3 of that of tetramethylammonium At higher doses the drugs also have anticholinergic properties Eighteen mg of PTMA given to a cat had nicotine paralyzing effects (Hunt 1926 and BTMA at 0.5 to 1.0 mg/kg showed antimuscarinic activity in the dog Long *et al* 1965) Wretlind (1950) reported curariform effects of PTMA at 0 5 mg/kg in the rabbit Hence PTMA and BTMA have a variety of actions at cholinergic sites in mammals at the doses used in the present study It seems plausible to assume that the toxicity of these drugs which appeared as respiratory distress at 7 5 mg/kg and higher doses in the present experiments is due to such actions Xylocholine (TM 10) has brief muscarinic and nicotinic actions even at doses near to those which affect sympathetic transmission (Exley 1957) However, bretylium βTM 10 and bethanidine show effects attributable to act

cholinergic sites, if any, only at very high concentrations (*bretylium*, Boura and Green 1959, Boura and Green 1965, *β TM 10*, McLean *et al* 1960a and b *bethanidine*, Boura and Green 1963). The guanidine derivatives of the Ph series lack noticeable muscarinic or nicotinic actions but, like guanethidine they have evident antinicotinic activity and neuromuscular blocking (Ph 881/7) effect *in vivo* (Hermansen 1963, Kadzielawa and Gumulka 1967, Hermansen pers comm). A relatively low activity at cholinergic sites of bretylium *β* TM 10 and bethanidine compared to those of PTMA and BTMA is also indicated in the rat by the lack of toxic symptoms even high doses (Lundberg 1969 and here). Hence, prominent activities at cholinergic sites are not a common feature among the delaying drugs.

The delaying activity of xylocholine (TM 10) seems to be much stronger than *that of β TM 10 (see Table I and Lundberg 1969). This is somewhat puzzling be*cause the two drugs are very closely related chemically. The difference in delaying action cannot be simply related to the stronger activity at cholinergic sites of TM 10 because the very potent delayer bretylium has only weak cholinergic and anticholinergic properties.

It has been discussed whether the release of sympathetic transmitter from adrenergic nerve endings is influenced by cholinergic mechanisms. Burn and Rand (1959 and 1965) have proposed a cholinergic link within the postganglionic adrenergic axon. It has also been suggested that acetylcholine released from a cholinergic neuron may act on a juxtaposed adrenergic neuron (Leaders 1963, Graham, Lever and Spriggs 1968). Acetylcholine and nicotinic drugs induce a release of noradrenaline from the isolated rabbit heart which is blockable by antinicotinic agents (Richardson and Woods 1959, Lindmar and Muscholl 1961). Recently there has also been shown a muscarinic inhibitory mechanism in the rabbit heart which affects noradrenaline release induced by nicotinic drugs or electrical stimulation of postganglionic sympathetic nerves (Lindmar, Loffelholz and Muscholl 1968, Loffelholz and Muscholl 1969). Hypothetically drug induced delay of the degeneration release of noradrenaline could be due to an action on a similar muscarinic inhibitory mechanism in the periorbital smooth muscle. However such a cholinergic hypothesis appears to be ruled out by the following findings. First bretylium delayed as efficient as PTMA although possessing less muscarinic activity. Second BTMA appeared to have less delaying activity than PTMA although being superior in muscarinic activity. Third, *atropine or methylatropine* did not inhibit the delaying action of PTMA.

Since the sympathomimetic effect of PTMA was not blocked by pretreatment with mecamylamine or atropine, not even this effect of the drug seems to be a cholinergic one. Both mecamylamine and hexamethonium lacked delaying effect when given alone. This fact appears to exclude the possibility that the degeneration release of sympathetic transmitter is due to a degeneration stimulation of nicotinic receptors in the nerve endings.

The sympathomimetic effect of bretylium or PTMA has been found to be increased in injections given around the start of the normal degeneration contraction

see Fig 2 This effect could be due to increased sensitivity—denervation supersensitivity—to catecholamines released from drug stimulated adrenals However it was shown above that an injection of bretylium or PTMA when the degeneration contraction had stopped and the nerve endings should be empty of transmitter and the denervation supersensitivity increased did not induce any excitatory action Hence the increased sympathomimetic effect of bretylium or PTMA is caused by changed conditions for the action of the drug at the degenerating nerve endings

I want to thank Professor E Barany for unfailing encouragement, Professor U Trendelenburg for instructive discussions and for showing me unpublished manuscripts Dr A F Green Beckenham Kent Dr K Hermansen, Copenhagen, Dr J P Long Iowa City, and Dr L J Tombleson Welwyn Garden City, Herts for supply of drugs and Mrs L Bennich Bjorkman and Mrs I Wersall for skillful technical assistance

This study was supported by a grant from Magnus Bergwalls stiftelse and by grant K69 14X 733-04C to Professor E Barany from the Swedish Medical Research Council

References

Boura A L A W G Duncombe and A McCoubrey The distribution of some quaternary

Pharmacol 1960 *15* 265—270

Boura A L A W G Duncombe R D Robson and A McCoubrey The distribution and excretion by cats of a new hypotensive drug n benzyl n n dimethyl guanidine *J Pharm Pharmacol* 1962 *14* 722—726

Boura A L A and A F Green The actions of bretylium adrenergic neurone blocking and other effects *Brit J Pharmacol* 1959 *14* 536—548

Boura A L A and A F Green Adrenergic neurone blockade and other acute effects caused by n benzyl n n -dimethylguanidine and its orto-chloroderivative *Brit J Pharmacol* 1963 *20* 36—55

Boura A L A and A F Green Adrenergic neurone blocking agents *Ann Rev Pharmacol* 1965 *5* 183—212

Burn J H and M J Rand Sympathetic postganglionic mechanism *Nature* (Lond) 1959 *184* 163—165

Burn J H and M J Rand Acetylcholine in adrenergic transmission *Ann Rev Pharmacol* 1965 *5* 163—182

Coats D A and N Emmelin Submaxillary and sublingual secretion in the cat during the degenerative phase following post ganglionic sympathetic denervation *J Physiol* (Lond) 1962 *161* 44—45P

Ehinger B Cholinesterases in ocular and orbital tissues of some mammals *Acta Univ Lund II* 1966 *2*

Exley K A The blocking action of choline 2 6 xylyl ether bromide on adrenergic nerves *Brit J Pharmacol* 1957 *12* 297—305

Graham J D P J D Lever and T L B Spriggs An examination of adrenergic axons around pancreatic arteriols of the cat for the presence of acetylcholinesterase by high resolution autoradiographic and histochemical methods *Brit J Pharmacol* 1968 *33* 15—20

system *J Pharmacol exp Ther* 1926 *28* 367—388

KADZIELAWA K. and W GLMULKA, Influence of new guanidine derivatives on ganglionic and neuromuscular transmission *Arch int Pharmacodyn* 1967 *170* 297—309

LANGER S Z, The degeneration contraction of the nictitating membrane in the unanesthetized cat *J Pharmacol exp Ther* 1966 *151* 66—72

LEADERS, F E, Local cholinergic adrenergic interaction Mechanism for the biphasic chrono tropic response to nerve stimulation *J Pharmacol exp Ther* 1963 *142* 31—38

LINDMAR R., K LOFFELHOLZ and E MUSCHOLL A muscarinic mechanism inhibiting the release of noradrenaline from peripheral adrenergic nerve fibers by nicotinic agents *Brit J Pharmacol* 1968 *32* 280—294

LINDMAR R and E MUSCHOLL Die Wirkung von Cocain, Guanethidin Reserpin Hexa methonium Tetracain und Psicain auf [illegible] *Naunyn-Schmiedebergs Arch exp Path I* [illegible]

LONG J P K C WONG and D L WITT, [illegible] trimethylamine and fluorobenzyl isomers

LOFFELHOLZ K and E MUSCHOLL, A m[illegible] evoked by postganglionic sympathetic nerve stimulation *Naunyn Schmiedebergs Arch exp Path Pharmak* 1969 *265* 1—15

LUNDBERG D Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat *Acta physiol scand* 1969 *75* 415—426

LUNDBERG D Interactions between inhibitors of the axonal amine pump and bretylium in the degeneration release of sympathetic transmitter *Acta physiol scand* 1970a *78* 503—521

LUNDBERG D Bretylium and the degeneration contraction of the sympathetically innervated periorbital smooth muscle in the rat *Acta physiol scand* 1970b *79* 411—422

LUNDBERG D The degeneration contraction of a sympathetically innervated smooth muscle in the rat after reserpine inhibition of monoamine oxidase or tyrosine hydroxylase *Acta physiol scand* 1970c *P0* 107—116

MALMFORS T and C SACHS Direct studies on the disappearance of the transmitter and changes in the uptake storage mechanisms of degenerating adrenergic nerves *Acta physiol scand* 1965 *64* 211—223

MCLEAN R A R J GEUS R J MOHRBACHER P A MATTIS and G E ULLYOT A series of 2 6-disubstituted phenoxyethyl ammonium bromides with true sympatholytic properties *J Pharmacol exp Ther* 1960a *129* 11—16

MCLEAN R A R J GEUS J PASTERNACK P A MATTIS and G E ULLYOT Pharmacology of trimethyl (2 (2 6 dimethylphenoxy) propyl) trimethylammonium chloride monohydrate compound 6890 or beta TM 10 *J Pharmacol exp Ther* 1960b *129* 17—23

PLUCHINO S L S VAN ORDEN III P R DRASKOCZY S Z LANGER and L TRENDELENBURG The effect of beta TM 10 on the pharmacological biochemical and morphological changes induced by denervation of the nictitating membrane of the cat *J Pharmacol exp Ther* 1970 *172* 77—90

RICHARDSON J A and E F WOODS Release of norepinephrine from the isolated heart *Proc Soc exp Biol N Y* 1959 *100* 149—151

SEARS M L and E H BARANY Outflow resistance and adrenergic mechanisms *Arch Ophthal* 1960 *64* 839—848

SMITH C B U TRENDELENBURG S Z LANGER and T H TSAI The relation of retention of norepinephrine H^3 to the norepinephrine content of the nictitating membrane of the spinal cat during development of denervation supersensitivity *J Pharmacol exp Ther* 1966 *151* 87—94

TREISTER G and E H BARANY The effect of bretylium on the degeneration mydriasis and intraocular pressure decrease in the conscious rabbit after unilateral cervical ganglionectomy *Invest Ophthal* 1970 9 343—353

WRETLIND K A J Studies on the pharmacological effects of trimethyl methylethyl and triethyl phenylammonium *Acta physiol scand* 1950 *20* 221—240

Acta physiol. scand. 1970. 80. 345—352

From the Department of Anatomy, University of Helsinki, Finland

Cytoplasmic Distribution of Endogenous and Exogenous 5-Hydroxytryptamine in Rat Peritoneal Mast Cells

By

STEN ERIK JANSSON

Received 3 March 1970

Abstract

JANSSON, S. E. *Cytoplasmic distribution of endogenous and exogenous 5-hydroxytryptamine in rat peritoneal mast cells.* Acta physiol. scand. 1970. *80.* 345—352.

The distribution of endogenous and exogenous 5-hydroxytryptamine (5-HT) in rat peritoneal mast cells was investigated by *in vitro* experiments using chemically 5-HT measurements in granules and supernatants after degranulation of the cells in a ion free medium and histochemically by the formaldehyde induced fluorescence in semi-thin Epon sections. About 20—30 % of the total amounts of 5-HT was found in the supernatant upon degranulation of a practically pure mast cell suspension. It is probably a solubilization artefact, because the formaldehyde induced fluorescence was always observed exclusively in the granules, while the intergranular cytoplasm was non-fluorescent. Even after incubation in exogenous 5-HT when the 5-HT content per cell was about 3 times that of normal cells no intergranular fluorescence was observed. Imipramine, reserpine and, to a lesser extent, ouabain at a concentration of 10^{-5} M inhibited the uptake but did not alter the normal granular distribution. This observation implies a reserpine resistant uptake of 5-HT by mast cell granules.

It has been shown by Day and Stockbridge (1964) that neoplastic ascites tumour mast cells take up 5-hydroxytryptamine (5-HT) both by an active process requiring energy and by passive diffusion. Normal peritoneal mast cells apparently handle the amine in a similar way (Jansson 1968, 1969). It was found that the uptake of 5-HT by normal mast cells during 1 hr from low exogenous concentrations of 5-HT was about 70 % over the endogenous 5-HT content. After incubation at high concentrations for several hours the 5-HT content increased up to 200 % above the original level (Jansson 1970). This raises the problem of the site of binding of exogenous 5-HT.

In normal mast cells, exogenous histamine, like endogenous histamine, is bound in the cytoplasmic granules (Thon and Uvnäs 1966, Cabut and Haegermark 1966). On the other hand, observations on ascites tumour mast cells indicate that an excess of exogenous 5-HT is located in the clear cytoplasm if the granular binding sites are saturated (Green and Furano 1962, Furano and Green 1964).

The present work was designed to study the intracellular distribution of 5-HT after incubation in the pres[illegible]

Material and methods

Rat peritoneal cells of which mast cells [illegible]

some experiments When the incubation conditions required a pure mast cell suspension, isolation was carried out by a modification of the method of Uvnas and Thon (1961) The density gradient used was one layer of 25 % Ficoll and centrifugation was carried out in the cold room at 4° C at about $350 \times g$ for 15 min

The incubation was performed in 8 ml siliconized glass culture tubes or in 10 ml plastic tubes Before incubation, the cells were preincubated in the medium for about 15 min at incubation temperature Thereafter, 1 ml of 5-HT dissolved in the modified Krebs Ringer albumin solution and pre-warmed to the incubation temperature was added to 1 0 ml of the cell suspension When the effect of drugs was studied, the cells were preincubated with the appropriate drug for 30 min at 37° C before 5-HT was added The incubations were interrupted by dilution with ice cold Krebs-Ringer solution and centrifugation at $2500 \times g$ for 10 min The cell pellet was then resuspended in two changes of fresh Krebs Ringer solution to wash out amine adsorbed onto the cells, and these were again collected by centrifugation

Degranulation of a pure mast cell population was performed according to Thon and Uvnäs (1966) After the cell pellet had been washed twice in Krebs Ringer solution, a further washing was carried out in 0 32 M sucrose adjusted to pH 6 9 with sodium hydroxide The cells were then resuspended in 1 0 ml 0 32 M sucrose and the suspension was frozen and thawed 3—6 times In some experiments degranulation was achieved by homogenization in a glass homogenisator Partially degranulated cells and aggregates of extracellular mast cell granules were collected by centrifugation at about $350 \times g$ for 3 min The supernatant was then centrifugated at about $2700 \times g$ for 30 min, which resulted in the sedimentation of free granules The 5 HT content in the final supernatant and the two cell debris pellets was determined according to the spectrophotofluorometric method of Weissbach (1961) as reported earlier (Jansson 1969)

The compartmentation of 5 HT in intact mast cells was studied using the formaldehyde induced fluorescence method for the histochemical demonstration of monoamines (Eranko 1955, 1967, Falck and Torp 1961) After incubation and washing one half of the cell suspension was transferred into conic plastic centrifuge tubes, while the other half was left in the incubation tubes The cells in the tubes were spun down as usual The cell pellet in the incubation tubes was prepared for biochemical determination of 5-HT, while the cell pellets in the centrifuge tubes were quenched in isopropane chilled with liquid nitrogen freeze-dried in vacuo for one or two days, treated with formaldehyde vapour under standardized conditions (Hamberger et al 1965) and finally embedded in epoxy resin (Epon 812) Dry 1 μ sections were cut with a LKB ultramicrotome

Results

On degranulation of a practically pure mast cell suspension by freezing and thawing or by homogenization in isotonic sucrose about 20—30 % of the total 5-HT content was found in the supernatant (Table I) The study whether this pool of 5-HT is normally located in the extragranular cytoplasm of intact mast cells the formaldehyde-induced fluorescence method was used

The amine fluorescence of normal rat mast cells can be examined in the thin Epon sections in great detail allowing observations of the amine site even inside the granules (Fig 2 There were differences in the fluorescence intensity not only between individual cells but also between individual granules of each cell In many normal mast cells non-fluorescent vacuoles were observed By subsequent staining with toluidine blue Fig 3) most of these vacuoles were shown to contain a pink-staining material while intact granules stained dark purple or blue The inter-

TABLE I Distribution of 5-HT in mast cells after incubation in exogenous 5-hydroxytryptamine

	5 HT, total µg/10⁶ MC	5-HT in supernatant % of total	Number of tests
Incubated 1 hr			
Control	0 290	20 7	3
5-HT 0 44	0 380	32 9	3
5-HT, 4 4	0 418	35 2	3
Incubated 2 hrs			
Control	0.240	17 5	3
5 HT, 0 22	0 485	20 5	3
5 HT, 4 4	0 744	25 2	3

Isolated mast cells were incubated at 37° C without 5-HT (control) or with 5-HT added After

granules and from the supernatant The table gives the total amount of 5-HT recovered and the amount of 5 HT found in the supernatant in per cent of total 5 HT

granular cytoplasm was non fluorescent, indicating that normally no 5 HT is to be found free in the cytoplasm

Incubation with 5 HT rendered the granules more intensely fluorescent but the intergranular cytoplasm was always non fluorescent after washing with Krebs-Ringer solution indicating that 5 HT taken up is bound by the granules (Fig 4)

This histochemical observation was supported by biochemical results On a percentual basis, about as much 5 HT was recovered from the supernatant after 5 HT incubation as from the supernatant of control cells not incubated in 5 HT

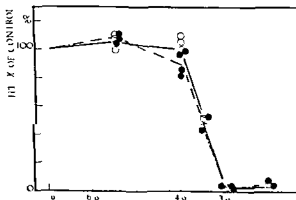


Fig 1 Effect of pH on the 5-HT content of mast cells incubated with 5 HT Incubations were carried out for 1 hr at 37° C with a 5-HT concentration of 0 44 µg/ml Thereafter the cells were washed twice by resuspension in two changes of fresh Krebs-Ringer solution and collected by centrifugation and then resuspended in 0 15 M phosphate buffer of different pH The extremely acidic pH values were achieved by addition of 0 1 N HCl The release of 5 HT was calculated in per cent of the 5-HT content of samples incubated at pH 7 0 with or without 5-HT respectively Each point represents one experiment Abscissa pH of the incubation medium in pH units Ordinate Mast cell 5 HT in per cent of control
○ Cells pre incubated without 5-HT, ● cells pre incubated with 5 HT

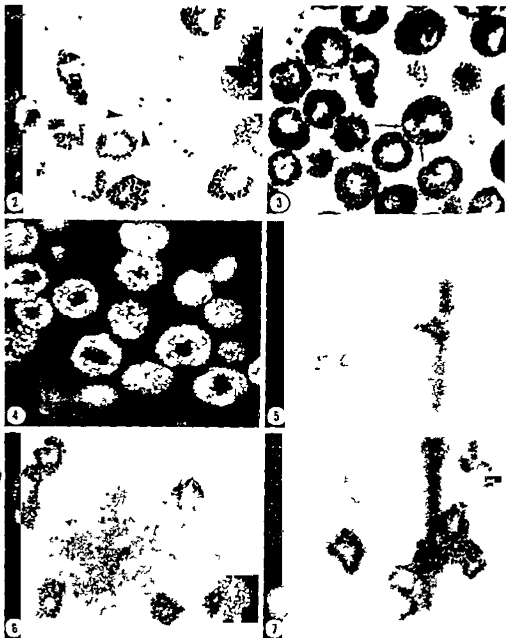


Fig 2 Formaldehyde induced fluorescence of rat peritoneal mast cells incubated for 1 hr at 37° C in Krebs-Ringer solution The fluorescence intensity differs between the individual cells and between the individual granules in the cells Note a few non fluorescent vacuoles (arrows) within some mast cells The intergranular cytoplasm seems to be non fluorescent 1 µ Epon section Magnification × 1850

Fig 3 The same section as in Fig 1 after staining with toluidine blue Intact granules stain dark purple or blue while a few granules stain pink or seem to be dissolved (arrows) The altered granules correspond to the non fluorescent vacuoles seen in Fig 1 Magnification × 1850

TABLE II Effect of drugs on the 5-HT content of mast cells after 5 HT incubation

Drug	Number of tests	Loss of 5 HT due to drug incubation, % of controls without drug	
		Normal cells	5 HT incubated cells
Chlorpromazine, 5×10 [illegible] M	6—6	—44 8	—40 5
Prenylamine, 5×10 [illegible] M	6—6	—44 5	—40 5
Reserpine, 10 [illegible] M	6—4	+10 4	—12 1

The cells were incubated for 1 hr at 37° C with or without 5-HT at 0 44 μg/ml After incubation

(Table I) This was also the case after incubation at high concentrations of 5 HT (4 4 μg/ml) for 2 hrs when the 5 HT content had increased about 200 % above the original level

By degranulating the mast cells, not by freezing and thawing but rather by incubation with chlorpromazine and prenylamine (for chemical and morphological data, see Jansson 1969), it was observed in the present study that these drugs affected the 5 HT content to the same extent whether or not the cells had been previously incubated with 5 HT (Table II) This observation further suggests a similarity in distribution and binding of 5 HT in normal and 5 HT incubated cells Reserpine on the other hand, caused an increased loss of 5 HT from cells previously incubated with 5 HT while the spontaneous loss of 5 HT from normal cells was slightly decreased by reserpine under the same conditions The significance of this observation cannot be discussed at present

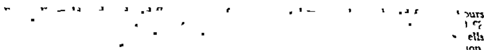


Fig 5 Formaldehyde induced fluorescence of mouse peritoneal mast cells Some faintly fluorescent cells are to be seen 1 μ Epon section. Magnification ×1850

Fig 6 Formaldehyde induced fluorescence of mouse peritoneal mast cells incubated for two hours at 37° C in 5 HT at 0 44 μg/ml The fluorescence intensity is clearly increased as compared with Fig 7 The intergranular cytoplasm is non fluorescent 5 HT content of the cells about 110 % higher than in Fig 5 1 μ Epon section Magnification ×1850

TABLE III Distribution of 5-HT in mast cells after incubation in exogenous 5-HT in the presence of drugs

	5-HT, total µg/10⁶ MC	5-HT in supernatant, % of total	Number of tests
Control	0 642	24 7	2
5-HT, 0 22 µg/ml	0 836	35 0	2
Imipramine, 10^{-5} M+ 5 HT, 0 22 µg/ml	0 616	34 7	4
Control	0 403	53 2	2
5-HT, 0 22 µg/ml	1 111	52 7	2
Ouabaine, 10^{-5} M+ 5-HT, 0 22 µg/ml	0 856	54 5	3
Control	0 739	29 8	4
5-HT, 0 22 µg/ml	1 143	36 3	4
Reserpine, 10^{-5} M+ 5-HT, 0 22 µg/ml	0 936	36 1	7

Isolated mast cells were incubated for 1 hr at 37° C without 5-HT (control), with 5-HT alone, or 5-HT and drugs, which had been added 30 min before the 5 HT After incubation the samples were washed and then frozen and thawed as explained in Table I

By incubating intact mast cells or isolated mast cell granules at different pH values no amine loss has been observed between pH 4 and 7, while the amine loss has been seen to be practically total at pH 3 or less (Åborg *et al* 1967, Jansson and Penttila 1969) In the present study, the 5-HT loss took place at pH 3 in both normal mast cells and 5-HT incubated and properly washed mast cells, while 5-HT was unaffected in both groups at higher values of pH (Fig 1)

Imipramine and reserpine at 10^{-5} M effectively depressed the uptake of 5 HT while ouabaine at 10^{-5} M had only a slight inhibiting effect in this respect However, none of the drugs induced any change in the intracellular distribution of 5 HT (Table III) Upon degranulation of the mast cells, equal proportions of 5 HT were found in the supernatant, whether the cells were incubated in the presence of the drug and 5-HT or with 5-HT alone Fluorescence microscopy also showed that the amine was situated in the granules

Because of the intense intrinsic fluorescence of rat mast cells, which obscured the slight increase in fluorescence intensity upon incubation with 5-HT in the presence of an inhibitor mouse mast cells were used in these histochemical studies As has been earlier reported (Eranko and Kauko 1965, Eranko and Jansson 1967, Jansson 1968) mouse mast cells normally exhibit a very faint fluorescence (Fig 5) Incubation in 5-HT rendered the mast cells intensively fluorescent, however, and a distinct granular distribution was observed (Fig 6) The fluorescence was weaker when drugs were present during the 5-HT incubation, but the fluorescence was still limited to the granules the intergranular cytoplasm being non-fluorescent (Fig 7)

Discussion

Thin epon sections are distinctly superior to conventional spread preparations in fluorescence microscopic study of mast cells A closer study of the fluorescent granules in spread preparations is possible only in degranulated or seriously flattened cells because intact mast cells in which there are several layers of granules exhibit a homogeneous and compact fluorescence By using thin epon sections it was observed that there were differences in fluorescence intensity not only between individual cells but also between individual intracellular granules During preparation of the present report Carlsson and Ritzen (1969) made the same observations using thin Amberlite sections

Thon and Uvnas (1966) suggested that histamine found in the supernatant after degranulation of mast cells in isotonic sucrose or water (about 30 % of total) might be due artificially to fractionation The present fluorescence microscopic observation that in normal intact mast cells the cytoplasm is essentially non fluorescent confirms this suggestion by indicating that at least a very high proportion of 5 HT is bound to the granules

The present morphological and biochemical results also indicate that incubation in 5 HT does not increase the intergranular concentration of 5 HT to any noticeable extent The results obtained by incubating at different pH values further indicate a similar binding of exogenous and endogenous 5 HT, i e to weak carboxylic groups (Åborg *et al* 1967)

These results are in contradiction with observations on neoplastic mast cells in which exogenous 5 HT seems to be stored in the intergranular cytoplasm as a soluble pool (Furano and Green 1964) This discrepancy can perhaps be explained according to Green (1966) who points out that neoplastic mast cells have only a few granules and thus the granular binding sites are more easily saturated than in normal cells

In the present study imipramine inhibited the uptake of 5 HT but did not alter the normal granular distribution of 5 HT in mast cells These results are in agreement with the present concepts of the mechanism of action of imipramine on the nervous tissue (see Carlsson 1966 Anden *et al* 1969) As in nervous tissue imipramine apparently depresses the uptake of 5 HT by mast cells at the cell membrane and leaves the uptake by granules unaffected

In the nervous tissue reserpine inhibits the uptake of amines by the granules being a very weak inhibitor of the membrane pump (see Carlsson 1966 Anden *et al* 1969) Thus amines taken up after treatment with reserpine accumulate in the cytoplasm (see Carlsson and Waldeck 1967)

In the present study reserpine depressed the uptake of 5 HT in mast cells but did not affect the normal granular distribution of this amine This observation together with the fluorescence microscopic findings implies a reserpine resistant uptake of 5 HT by the granules in mast cells This might be a physico chemical process due to the unique chemical composition of the mast cell granules (see Åborg *et al* 1967, Bergqvist 1969)

References

ABORG CH. J NOVOTNÝ and B UVNÄS, Ionic binding of histamine in mast cell granules *Acta physiol scand* 1967 69 276—283

ANDEN, N E, A CARLSSON and J HAGGENDAL, Adrenergic mechanisms *Ann Rev Pharmacol* 1969 9 119—134

BERGQVIST, U, Chemistry of mast cell granules *Acta physiol scand* 1969 Suppl 330

CABUT, M and Ö HAEGERMARK Uptake, storage and release of histamine by rat peritoneal mast cells in vitro *Acta physiol scand* 1966 68 206—214

[illegible] nous [illegible] Rosell [illegible] nerve fibres of reserpine treated mice *J Pharm Pharmacol* 1967 19 182—190

CARLSSON S A and M RITZEN, Mast cells and 5 HT Intracellular release of 5 hydroxytryptamine (5 HT) from storage granules during anaphylaxis or treatment with compound 48/80 *Acta physiol scand* 1969 77 449—464

DAY M and A STOCKBRIDGE The effects of drugs on the uptake of amines by mast cells *Brit J Pharmacol* 1964 23 405—419

ERÄNKÖ O, Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster *Acta endocr (Kbh)* 1955 18 174—179

ERÄNKÖ O, The practical histochemical demonstration of catecholamines by the formaldehyde induced fluorescence *J roy micr Soc* 1967 87 259—276

ERÄNKÖ O and S E JANSSON Uptake of monoamines by mouse peritoneal mast cells in vitro *Acta physiol scand* 1967 70 449—450

ERÄNKÖ O and L KALKO Uptake of monoamines by mesenteric mast cells of the mouse *Acta physiol scand* 1965 64 283—284

ERÄNKÖ O and L RÄISÄNEN In vitro release and uptake of noradrenaline in the rat iris In *Mechanisms of release of biogenic amines* (Eds U S von Euler S Rosell and B Uvnäs) Pergamon Press Stockholm 1966

FALCK B and A TORP A fluorescence method for the histochemical demonstration of monoamines in the adrenal medulla *Med exp (Basel)* 1961 5 429—432

FURANO A V and J P GREEN The compartmentation and elimination of ^{14}C-histamine by neoplastic mast cells in culture *Biochim biophys Acta (Amst)* 1964 86 596—603

GREEN J P Synthesis, uptake and binding of histamine and 5 hydroxytryptamine in mast cells In *Mechanisms of release of biogenic amines* (Eds U S von Euler S Rosell and B Uvnäs) Pergamon Press London 1966

GREEN J P and A V FURANO Two pools of amines in neoplastic mast cells *Biochem J Pharmacol* 1962 11 1049—1053

HAMBERGER B T MALMFORS and CH SACHS The standardization of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholmines *J Histochem Cytochem* 1965 13 147—148

JANSSON S E Effect of pH and temperature on the uptake of monoamines by mouse peritoneal mast cells in vitro *Acta physiol scand* 1968 73 196—203

JANSSON S E Effect of chlorpromazine, mepyramine prenylamine and reserpine on 5 hydroxytryptamine content and fine structure of rat peritoneal mast cells incubated in vitro *Acta physiol scand* 1970 78 420—430

JANSSON S E Uptake of 5 hydroxytryptamine by rat peritoneal mast cells in vitro *Acta physiol scand* 1970 79 481—492

JANSSON S E and A PENTTILA Effect of pH temperature osmolarity and drugs on the 5 hydroxytryptamine content and light and electron microscopic structure of rat peritoneal mast cells in vitro *Exp Cell Res* 1969 54 367—376

THON I L and B UVNÄS Mode of storage of histamine in mast cells *Acta physiol scand* 1966 67 455—470

UVNÄS B and I L THON Evidence for enzymatic histamine release from isolated rat peritoneal mast cells *Exp Cell Res* 1961 23 45—57

WEISSBACH H The estimation of Serotonin in biological material *Stand Meth clin Chem* 1961 4 197—203

Acta physiol. scand. 1970. 80. 353—359

From the Surgical Departments I and III, Sahlgren's Hospital, University of Gothenburg
Sweden

The Effect of Periarterial Nerve Stimulation on the Jejunal and Ileal Motility in Cat

By

J. KEWENTER, P.-E. PAHLIN and B. STORM

Received 5 March 1970

Abstract

KEWENTER, J., P. E. PAHLIN and B. STORM. *The effect of periarterial nerve stimulation on the jejunal and ileal motility in cat.* Acta physiol. scand. 1970. *80.* 353—359.

The effect of nervous stimulation of the periarterial nerve trunks along the superior mesenteric [illegible]

There seems to be no difference in the opinion about the presence of adrenergic inhibitory fibres to the small intestine in the periarterial nerve trunks along the superior mesenteric artery. Several investigations have been performed preferably *in vitro* where this has been shown (*e.g.* Finkleman 1930, Boyd, Gillespie and MacKenna 1962). However, the presence of true parasympathetic cholinergic fibres in these nerves has been questioned by some authors (Day and Rand 1961), while others consider that the excitatory responses obtained at stimulation of these nerves after administration of adrenergic blocking agents are due to an activation of parasympathetic fibres. A special interest has during the last years been focused upon the functional significance of these nerves as they have to be divided at transplantation of the small intestine. Thus, Ballinger, Christy and Ashby (1962) showed in dogs that division of these nerve trunks induced protracted diarrhea. This effect of denervation of the small intestine was not possible to confirm in cats (Kewenter and Storm 1969).

The aim of the present investigation has been to examine the functional significance on the small intestine motility *in vivo* of the nerve fibres running in nerve trunks along the superior mesenteric artery.

Methods

Experiments were performed on 10 cats of both sexes The animals were anesthetized

valve The distal and proximal division respectively was performed so that each loop was al 6—8 cm long The loops were thus isolated from adjacent parts of the small intestine but ot wise left *in situ* Great care was taken not to damage the vascular and nervous supply in mesenteric pedicle of the prepared loops The content in the intestinal segments was remo and the distal end of each loop was connected to a glass tube which by way of a rubber t and an adaptable pressure reservoir led to a piston recorder The systems were filled

The nerve trunks along the superior mesenteric artery were cautiously isolated from superior mesenteric artery and cut proximally The distal end was placed on a coil form bipolar silver electrode introduced into the abdomen via a small separate opening The isol electrode was connected to a Grass Stimulator S 4 Stimulations of 6—16 imp/sec at 2 to 20 and a pulse duration of 0 05 to 5 0 msec were used

Atropine (1 mg/kg) and guanethidine (1 mg/kg) were infused into the inferior caval v by means of a fine catheter introduced into one of the femoral veins

Results

In general the proximal part of the small intestine responded with excitation of pe arterial nerve stimulation more readily than the distal part where inhibitory mot responses were more easily induced However in most experiments it was possible induce both excitatory and inhibitory motor responses according to the stimul strength Clearcut inhibitory motor responses were induced when voltage and/ pulse duration were increased as compared to those necessary to get an excitato motor response At a constant voltage and frequency it was possible first to induce motor response followed by an immediate inhibition of the motor activity when th pulse duration was increased (Fig 1A) Inhibitory motor responses were also po sible to obtain with the higher pulse duration without any previous excitation of th motor activity (not illustrated in the Fig)

In some experiments periarterial nerve stimulation induced an excitatory mot response in the jejunum and an inhibitory response in the ileum Such an exper ment is illustrated in Fig 2A When voltage was increased from 8 V to 20 V a clea cut inhibitory response was obtained in the ileum but not in the jejunum The re verse response was never obtained indicating that the lower part of the small in testine is more easily inhibited at periarterial nerve stimulation

The effect of variation in stimulation frequency with a constant voltage and pul duration was also investigated It is well known that the discharge rate in the auto nomic nervous system hardly ever exceeds some 10 imp/sec (Folkow 1955) At highe frequencies there is an overflow of transmitter from the vasoconstrictor fibres whic will cause a false inhibitory motor response in the small intestine (Celander 1959 Kock 1959) This fact is illustrated in Fig 3A At a constant voltage and pulse dura

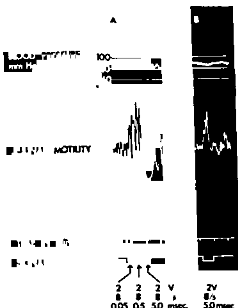


Fig 1

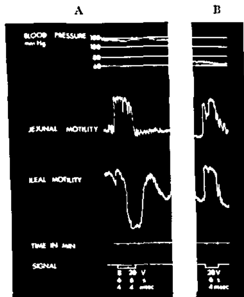


Fig 2

Fig 1 Chloralose Effect of periarterial nerve stimulation on the jejunal motility and blood pressure at low and high pulse duration (A) B illustrates the effect of stimulation with the high pulse duration after administration of guanethidine (1 mg/kg)

Fig 2 Chloralose Effect of 'weak' and "strong' efferent stimulations of the periarterial nerve on jejunal and ileal motility and blood pressure (A) B Periarterial nerve stimulation with the strong current after administration of guanethidine (1 mg/kg)

tion 8 imp/sec induced a motor response in the ileum which was changed to an inhibitory response, when the frequency was increased to 16 imp/sec In these experiments this higher frequency never induced any excitatory motor responses, although at frequencies below 10 imp/sec such were elicited at the same voltage and pulse duration Periarterial nerve stimulation never influenced the blood pressure significantly although the stimulus strength and duration was considerably varied

In all experiments the periarterial nerves were stimulated after i v administration of 1 mg/kg of guanethidine in order to block the adrenergic nerve fibres to the intestine as described by Boyd Gillespie and MacKenna (1962) Intestinal inhibitory responses could then no longer be induced by electrical stimulation Instead jejunal and ileal excitatory motor responses were obtained with the same stimulation parameters that before administration of guanethidine had elicited prompt inhibitory responses (see Fig 1B 2B and 3B) Excitatory responses were seen in bot jejunum and ileum in all experiments except in the ileum in one cat In all these experiments an i v infusion of adrenaline induced an inhibition indicating that the absence of inhibitory responses in jejunum and the ileum to periarterial nerve stimulation could not be ascribed to any lack of intestinal tone

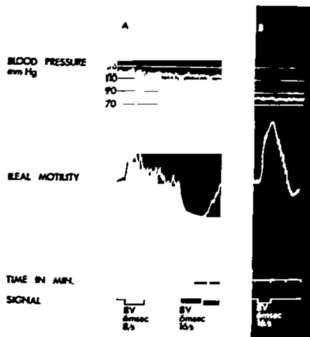


Fig 3 Chloralose Effect of efferent periarterial nerve stimulation on the ileal motility and blood pressure at physiological and supra physiological frequencies (A) B Periarterial nerve stimulation on the supra physiological frequency after administration of guanethidine (1 mg/kg)

Discussion

In previous investigations it has been said that two factors determine the nature of the small intestine motor responses namely the tone level of the intestine and the stimulation frequency used (Garry 1957, Harn 1963) Low tone was said to favour excitatory motor responses and high tone inhibitory motor responses However, at least with respect to the gastric smooth muscles Harper, Kidd and Scratcherd (1959) and Martinson (1964 were unable to support such views The present experiments do not confirm this view either as both excitatory and inhibitory motor responses could be elicited from the same level of intestinal tone This does not deny that it was somewhat difficult to induce clearcut inhibitions of intestinal motility when the tone was initially low just as it could be difficult to increase the intestinal motor activity when this was very intense

The nature of the motor responses was only due to the stimulus strength Thus a high voltage and/or pulse duration induced inhibitory responses while a lower stimulus strength induced excitatory responses

In the majority of experiments performed on intestinal motility where the autonomic nerves have been stimulated both in vivo and in vitro rather high frequencies have been used Thus Garry and Gillespie (1955) found in experiments performed on rabbit colon in vitro that maximal excitatory motor responses was obtained around 10 imp/sec at parasympathetic stimulation and when the sympathetic outflow was stimulated with frequencies of 100 imp/sec or higher, maximal inhibitory motor responses were elicited This is in accordance with the fact that in almost all investiga-

tions performed where the autonomic nerves to the intestine have been stimulated with high frequencies, such stimulations produce inhibitory motor responses However, the discharge rate in the autonomic nervous system is normally below 10 imp/sec (see e g Folkow 1955) and an overflow of the transmitter occurs at the vasoconstrictor nerve endings at higher rates, which induces an inhibition of the intestinal smooth muscle cells (Celander 1959, Koch 1959) The inhibitory motor responses obtained at stimulation at 'supraphysiological' rates can therefore hardly be taken as an evidence that the nerves convey specific inhibitory fibres to the investigated part of the small intestine However, the inhibitory responses obtained in the present investigation were all obtained at low frequencies and can therefore hardly be due merely to an overflow of the transmitter from concomitantly activated vasoconstrictor fibres With the same relatively low frequencies, e g 6—8 imp/sec, it was possible to induce excitatory as well as inhibitory motor responses by changing the voltage and/or pulse duration, indicating that the periarterial nerves contain two different sets of nerve fibres mediating excitatory and inhibitory impulses to the small intestine The inhibitory responses were obtained at a stimulus strength about 2 to 10 times those necessary to elicit excitatory responses

Most previous authors dealing with the fibre sets in the mesenteric periarterial nerves have used a rather constant voltage and pulse duration in their experiments (Gillespie and MacKenna 1961, Boyd Gillespie and MacKenna 1962) This fact may explain why they have not been able to demonstrate the pressure of two distinctly different groups of fibres, mediating excitation and inhibition respectively, unless they have treated the animals with reserpine bretylium or guanethidine Guanethidine abolished the inhibitory responses in the present experiments both in the jejunum and the ileum Excitatory responses of the intestinal segments were obtained in all experiments at periarterial nerve stimulation after administration of this drug

Burn and Rand (1960) and Day and Rand (1961) have claimed that the post ganglionic sympathetic nerves may themselves be cholinergic and that nerve impulses liberate acetylcholine which in turn releases noradrenaline and the responses are therefore adrenergic Guanethidine blocks the release of noradrenaline and acetyl choline liberated at the sympathetic nerve endings by the nerve imp lse diffuses to

[illegible] obtained at periarterial nerve stimulation after administration of guanethidine was not due to activation *of the true* parasympathetic fibres However depending on the stimulus strength it was in the present experiments *in vivo* possible to induce clearcut excitatory and *inhibitory* responses without administration of sympathetic blocking agents and the excitatory responses were induced when voltage and the pulse duration was kept low *These* results therefore hardly support the concept of Day and Rand but rather that *of* Finkleman (1930 and Boyd Gillespie and MacKenna (1962) that the periarterial mesenteric nerves contain two distinctly different sets of excitatory and inhibitory *fibres*

In the present experiments inhibitory responses were more [illegible] larly obtai

the ileum than in the jejunum at the periarterial nerve stimulation In some experiments the same nerve stimulation induced an excitatory response in the jejunum and an inhibitory response in the ileum Celander (1959) and Koch (1959) found in the cat that splanchnic nerve stimulation did not induce any inhibitory responses in the jejunum with physiological frequencies Kewenter (1965) confirmed this finding and further showed that inhibitory responses were easily obtained in the ileum at splanchnic nerve stimulation with low physiological frequencies The present results also suggest a quantitative difference in the extent of adrenergic nerve supply to the upper and lower parts of the small intestine Thus, the lower parts of the small intestine seem to have a stronger adrenergic inhibitory innervation than the upper part of the gut Nylander and Wikstrom (1967) found in experiments in rat where they investigated the propulsion through the small intestine, that the passage through the proximal part of the small intestine was more rapid than through the distal part This statement is further supported by the well known fact that the rate at which a contrast medium or a telemetering capsule traverses the small intestine is higher in the upper than in the lower part of the small intestine (see Connell 1961) It can be concluded that the entire small intestine is innervated by adrenergic inhibitory fibres which are involved *e g* in spinally mediated intestino-intestinal inhibitory reflexes (Kuntz and Saccomanno 1944 Johansson and Langstone 1964) Besides these inhibitory nerve fibres which to a great extent run in aberrant preganglionic connections to the coeliac ganglia rather than in the splanchnic nerves proper the lower part of the small intestine is innervated by adrenergic fibres mediated through the splanchnic nerves The functional significance of this latter group of fibres is not fully understood but it is possible that they convey a tonic sympathetic control of supraspinal origin exerting its influences mainly on the ileum (Kewenter 1965)

This work was supported by grants from Semper Fond for Naringsforskning

References

BALLINGER W F M G CHRISTY and W B ASHBY Autotransplantation of the small intestine the effect of denervation *Surgery* 1962 *52* 151—164

BOYD G J S GILLESPIE and B R MACKENNA Origin of the cholinergic response of the [illegible] hetic [illegible]

B[illegible] of [illegible] 196[illegible]

CELANDER O Are there any centrally controlled sympathetic inhibitory fibres to the musculature of the intestine *Acta physiol scand* 1959 *47* 299—309

CONNELL A M The motility of the small intestine *Postgrad Med J* 1961 *37* 703—71[illegible]

DAY M D and M J RAND Effect of guanethidine in revealing cholinergic sympathetic fibres *Brit J Pharmacol* 1961 *17* 245—260

FINKLEMAN B On the nature of inhibition in the intestine *J Physiol* (Lond) 1930 *70* 14[illegible]—157

FOLKOW B Nervous control of the blood vessels *Physiol Rev* 1955 *35* 629—663

GARRY R C and J S GILLESPIE The response of the musculature of the colon of the rabbit to stimulation *in vitro* of the parasympathetic and of the sympathetic outflow *J Physiol* (Lond) 1955 *128* 557—576

GARRY, R C, Innervation of abdominal viscera *Brit Med Bull* 1957 *13* 202—20[illegible]

GILLESPIE J S and B R. MACKENNA The inhibitory action of the sympathetic nerves on the smooth muscle of the rabbit gut its reversal by reserpine and restoration by catecholamines and by DOPA *J Physiol* (Lond) 1961 *156* 17—34

HARRY G L V Responses of muscles of cat small intestine to autonomic nerve stimulation *Amer J Physiol* 1963 *204* 352—358

HARPER A A C KIDD and T SCRATCHERD Vago-vagal reflex effects on gastric and pancreatic secretion and gastrointestinal motility *J Physiol* (Lond) 1959 *148* 417—436

MARTINSON J The effect of graded stimulation of efferent vagal nerve fibres on gastric motility *Acta physiol scand* 1964 *62* 256—262

NYLANDER G and S WIKSTROM Gastric emptying and propulsive intestinal motility following partial gastric resection gastroenteroanastomosis and abdominal trunk vagotomy in the rat *Acta chir scand* 1967 *133* 41—54

Acta physiol. scand. 1970. 80. 360—369

From the Department of Physiology, University of Helsinki, Finland

Localization of a Microelectrode Tip in Muscle Cell

A light and electron microscopic study

By

J NICKELS

Received 16 March 1970

Abstract

NICKELS J *Localization of a microelectrode tip in muscle cell A light and electron microscopic study* Acta physiol scand 1970 80 360—369

A method is described for accurate localisation of a recording glass microelectrode in muscle cell and subsequent histochemical staining of the myoneural junction of the cell with cholin esterase and finally for local [illegible] picture Microelectrodes were f[illegible] the microelectrode at the recor[illegible] cent acetate buffered (pH 5 6) [illegible] driven electrophoretically from [illegible] into the muscle cell The whit[illegible] $(NH_4)_2S$ The size of the sediment was 5—20 μ It was seen clearly under the [illegible] scope This method did not affect the histochemical stainability of the motor end plate As the sediment contained heavy metals it was visualised also in the electron microscopic picture after further fixation for one hour in buffered glutaraldehyde The sediment was visible also in the Epon resin block and this helped the trimming of the specimen The sediment consisted in a normal electron microscopic picture of rough heavy metal grains

Accurate localisation of the microelectrode tip in the tissue to be recorded has always been difficult No really good method has been evolved so far although several different applications have been suggested Methods have varied according to the tissue under examination and whether glass or metal electrodes are used

The recording point of steel microelectrodes can be localised through Prussian blue reaction by releasing ferroions with direct current from the electrode into the tissue A mark of about 200 μ was obtained in this way at the recording point (Scheibel and Scheibel 1956) Hubel (1959) simply used an electrocoagulation lesion of 100—200 μ to localise tungsten microelectrodes

Ordinary metal electrodes were localised by Robinson (1962) by measuring the impedance of the different parts of the brain He was able to determine the location of the electrode better from the impedance variation than with the ordinary stereotactic methods He suggested that the method has possible applications also in work with microelectrodes

Iontophoresis has usually been employed in the localisation of glass microelectrodes in the central nervous system to drive various ions into tissue. Bultitude (1958) used the Prussian blue reaction, like Rayport (1957) who was able to mark cortical neurons. The iron was in a complex form inside the microelectrode. On the other hand, Sokolov, Arakelov and Levinson (1966) used ordinary $FeCl_3$ in their microelectrodes and were able to mark individual neurons of the Linnea stagnalis ganglion. According to Hyvarinen (1966), the Prussian blue reaction is poor at any rate for extracellular work as the intensity of the sediment is too weak. Galifret and Szabo (1960) used complex copper as the anion and marked several recording points in the same preparation. The sediment area measured 50—100 μ. According to the literature, hardly any use has been made of this method.

By filling the microelectrodes with saturated methylene blue Thomas and Wilson (1966) were able to mark individual neurons. They had previously (1965) used fast green FCF for marking after extracellular recording. The latter method was improved by Grossman, Whiteside and Hampton (1969).

Stere *et al.* (1968) combined with methylene blue staining a new histologic chrushing technique which made it possible to examine the marked cell in its entirety.

Among the most recent methods in the region of the central nervous system are the one suggested by Stretton and Kravitz (1968), in which Procion Yellow M4RS fluorescence dye is used as the marking agent, and the Alcian blue 8GX dye method used by Lee, Mandl and Stean (1969).

For retinal recordings the tip of the microelectrode has been localised iontophoretically with crystal violet (MacNichol and Svaetichin 1958) and Ag ions (Oikowa, Ogawa and Motokowa 1959).

Numerous microelectrode recordings have been performed extraneurally using similar methods to those applied in the central nervous system for marking the position of the microelectrode tip. Villegas (1962) localised the potential difference area in the gastric mucosa of frogs by using microelectrodes filled with 3 M lithium carmine and studying under a phase contrast microscope the dye that had been driven electrophoretically into the tissue. The same dye was used by Ussing and Windhager (1964) in their study of the potential difference in the epidermis compared with the dermis. Using microelectrodes filled with Chicago blue 6-B Revel and Sheridan (1968) studied the brown fat of the mouse and its potential characteristics.

Muscle cell and the myoneural junction have been studied extensively by the microelectrode technique. Nerve muscle preparations from the frog or rat are usually used for the investigations. Such preparations can be placed in a nutrient medium chamber under the preparation microscope. The muscle cell studied can be identified later with the help of *e.g.* the nerve branches seen in the preparation microscope. In addition the myoneural junction can be stained histochemically. This method was used by Katz and Miledi (1964) but its drawback is that on moving away from the fine nerve branches that innervate the muscle cells a distinct point of attachment for the later localisation of the muscle cell is no longer found.

Nor is the method suitable for *in vivo* work No dye marking method has been described for muscle cells, obviously because the dyes suitable for the central nervous system are diffused rapidly in a large muscle cell, making it impossible later to localise the site of the tip in the muscle under the light microscope Further, no method has been reported in the literature which could be used to localise the cell to be recorded and the position of the electrode tip and which would permit study also at the electronmicroscopic level

This paper presents a method by which the precise recording point of the microelectrode can be identifeid after recording *in vivo* and the corresponding myoneural junction can be stained histochemically after the localisation procedure The muscle cell and recording point can be identified also at the electron microscopic level by means of the sediment obtained A preliminary report was published earlier (Nickels 1968 1969)

Methods

Muscles of the frog extremities were used in the preliminary experiments and rat muscle for the continuation studies The rats were fixed on their back on a cork plate after Nembutal® (40—50 mg/kg) narcosis The tibialis anterior muscle was dissected free and ordinary microelectrode recordings were made The microelectrodes were glass capillaries with a tip diameter of about 0 5 μ the resistance was 5—10 MΩ when the capillaries were filled with 1 1 M of $K_4Fe(CN)_6$ and 1 5 M of Na_2HPO_4 The muscle was moistened continuously with Ringers solution for mammals during the recording After the recording a 1 1 mixture of 0 1 M $Pb(NO_3)_2$ and 5 per cent acetate buffered (pH 5 6) glutaraldehyde was applied around the microelectrode The anions were then driven into the muscle cell using about 2 μA DC for 5—10 sec The microelectrode was raised slowly during electrophoresis White sediment formed in the muscle cell around the microelectrode tip and it was turned dark by 1 % $(NH_4)_2S$ A superficial section of 50—100 μ was made of the muscle by a freezing microtome or the muscle cell itself which was identified with the help of the sediment was freed from its environment To examine the motor end plate it was stained histochemically in the manner described by Joo Sávay and Csillik (1965) for the demonstration of cholinesterase

For electronmicroscopic study the muscle was fixed further for 1 hr in phosphate buffered (pH 7 4) 5 % glutaraldehyde This was followed by routine electronmicroscopic treatment osmium fixation for 30 min dehydration with alcohol and embedding in Epon and preparation of sections Uranyl acetate was used for after staining The microscopic examination was performed under a Hitachi HS7S electron microscope

Results

Light microscopy

It was possible to perform the microelectrode recordings normally from the muscle cell (Fig 1 a) The size of the marker sediment that originated ranged from 5 to 20 μ (Fig 1 b) Success depended largely on how accurately the superficial fascia was dissected free for otherwise the $Pb(NO_3)_2$ was unable to affect the muscle cell itself and reacted with the connective tissue Frog muscle is easier to treat in this respect as it has relatively less connective tissue than rat muscle The sediment that formed in a frog muscle cell was often more dispersed (Fig 2) As the microelectrode was raised during electrophoresis sediment often formed close to the membrane or in the membrane itself It was then possible to establish also deeper in the muscle cell the track left by the microelectrode and the coagulation area of its tip (Fig 3)



Fig 1 a Miniature end plate potentials recorded by microelectrode which are caused by the spontaneous liberation of acetylcholine at the myoneural junction Calibration 2 msec and 1 mV

Fig 1 b The myoneural junction from which the recording in Fig 1 a was made The arrow indicates the recording site Bar indicates 20 μ

The fixing agent obviously prevented the track from closing Marking sometimes failed because the sediment stuck to the microelectrode tip and broke off from the muscle cell when the electrode was raised

Histochemical staining of the myoneural junction itself succeeded readily if the junction was above the muscle cell However it is usually below the muscle cell where the reacting substances are unable to act upon it during a normal incubation

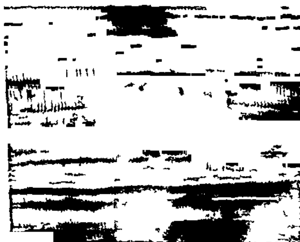


Fig 2 Frog muscle cells ... one of which ... widespread marking sediment is seen Bar indicates 30

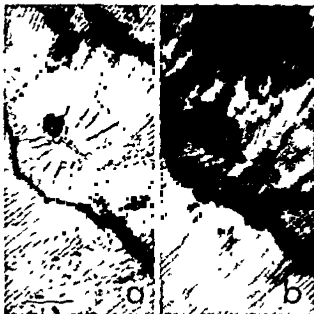


Fig 3 a Rat muscle cells one d which shows sediment at the recording point

Fig 3 b The same muscle cell. The track left by the microelectrode is visible deeper down. The arrow indicates the coagulation area of the microelectrode tip Bar indicates 20 μ

period It was therefore imperative to limit the superficial sections to the thickness of two cell layers or dissect the muscle cell free The hole left by the tip of the microelectrode in the muscle cell membrane was sometimes visualised without preceding electrophoresis (Fig 4) This was obviously because the lead reacted more readily with a pierced than with an intact membrane Treatment of the muscle cell with glutaraldehyde prior to electrophoresis prevented the occurrence of any major deformity Without this treatment the muscle cell broke easily when the current was applied It was sometimes possible to see the myoneural junction without the histochemical staining mentioned above This was obviously due to the so-called lead reaction when lead reacts with the sulphydryl groups in the subneural space (Savay and Csillik 1950 the myoneural junction is visualised after $(NH_4)_2S$ treatment



Fig 4 Muscle cell inside which there has been a microelectrode. The place has not been marked with sediment but later when treating the muscle $Pb(NO_3)_2$ has reacted with the edges of the gap causing an identifiable hole



Fig 5 a Typical marking sediment seen under the light microscope Bar indicates 20 μ
Fig 5 b The same sediment in an Epon resin block

This was observed only if the motor endplate was located above the muscle surface

Electron microscopy

The black sediment which formed in the muscle was easy to detect in the light microscope and in the Epon resin block (Fig 5) which greatly facilitated the trimming of the section itself When the actual sediment site was cut scratches often originated possibly from the heavy-metal sediment itself or because the electrophoresis current broke the microelectrode tip and glass fragments entered the muscle cell and scratched the glass knife Fairly large heavy metal grains of coarse structure (Fig 6) were seen in the electronmicroscopic picture of the sediment The grains were located chiefly between the myofibrils in the sarcoplasm The sediment was



Fig 6 Electronmicroscopic pictures of heavy metal marking sediment inside muscle cell Bar indicates 1 μ

clearly distinguishable from the glycogen granules which were smaller and of regular shape Corresponding heavy metal particles were not demonstrated elsewhere in the same muscle cell nor in other muscle cells

Discussion

The diameter of the muscle cells varies fairly considerably even in the same muscle There is evidence that the electrical properties of the muscle cell membrane are dependent on the thickness of the muscle cell (Rosenfalck 1969) For instance the rise time of the miniature end plate potentials is shorter in thin muscle cells and their amplitude is greater (Katz and Thesleff 1957) Comparison of the electrophysiologic properties of an individual muscle cell and myoneural junction with the anatomic characteristics of the cell requires unless the recording is being made from a single cell preparation the identification of the cell in question from among hundreds of other cells after the recording Katz and Miledi (1964) sought to solve the problem by using a myoneural preparation in vitro under the microscope However in vitro findings do not always agree with results obtained in vivo In fact Krnjevic and Miledi (1958 showed that the intracellular sodium concentration in an isolated rat phrenic nerve preparation after a 5 hr experiment was the same as that of the nutrient medium of the preparation When the same muscle was used in vivo only small changes occurred in the intracellular sodium and potassium concentrations. The most reliable electrophysiologic results are obtained when the cell is in its natural environment To identify the cell after the recording it must be possible to mark the recording point The recording electrode must thus function at the same time as a marking electrode However this must not change its recording properties and of course must not affect the tissue under examination during the recording

The mark must lie within the area of the tip and must be as small as possible but clearly visible under the microscope It ought to be possible to mark several recording points with the same microelectrode When the myoneural junction is under examination the method must not affect the histochemical properties of the motor end plate and if the sediment is to be identified also from the electronmicroscopic picture it must be of heavy metal Solutions to these problems were sought in the present study

Of methods published earlier the vital dyes methylene blue and Janus green B were experimented with but the concentration of the dye after electrophoresis was very small in the recorded area becaue of its rapid diffusion over a wide area in the muscle cell It is known too that these two dyes are potent acetylcholinesterase inhibitors (Renz 1940) and hence are probably not suitable for study of the myoneural junction in which acetylcholinesterase is an essential component although the diffusion from the microelectrode tip to the muscle cell during the recording is certainly very small Heavy metals produce insoluble dark sediments with $(NH_4)_2S$ The method employed by Sokolov Arakelov and Levinson (1966) in which the microelectrode was filled with 2 5 M $FeCl_3$ was experimented with However it appeared that the impedance of the microelectrode when it was inserted in the muscle cell rose in a few seconds from 5 MΩ to infinite This was obviously attributable to the reaction of Fe ions with the tissue proteins which choked the tip of the capillary The complex form of heavy metal was therefore decided upon and $K_4Fe(CN)_6$ with Na_2HPO_4 as the other electrolyte were selected The former is indifferent to tissue and has been used in renal function tests on man (Stieglitz and Knight 1934) The latter is a normal ion of the organism Both agents function as electrolytes in microelectrode recording Na_2HPO_4 increases the solubility of $K_4Fe(CN)_6$ in water and at the same time appreciably inhibits its precipitation which occurs with time The anions of both substances form with lead an insoluble white sediment which can be turned dark with $(NH_4)_2S$ Nor do the other reagents employed in the marking procedure change the histochemical staining of cholinesterase in the myoneural junction $Pb(NO_3)_2$ is also used in the incubation fluid in this histochemical staining procedure and $(NH_4)_2S$ is used in the after treatment

The sediment formed is easy to visualise under the light microscope and the method does not cause any appreciable changes in the muscle cell itself though the electrophoresis current does cause a slight contraction in the region of the microelectrode tip A histochemically stained myoneural junction also displays normal details (see Fig 1) The success rate is approx 50 per cent Accurate dissection free of the fasciae above the muscle cell is important This is clearly more difficult to achieve in an older rat than in young rats or in frogs A drawback of the method is that also other muscle cells are fixed close to the labelled muscle cell making it impossible to use them for recording

The sediment seen in the preparation microscope was very useful in the electron microscopic work as it made it easy in a multicell preparation to identify the muscle cell concerned when cutting the sections and made it possible for the worker to



Fig 7 Histochemical staini a myoneural junction The . indicates the terminal part c axon with its mitochondria. histochemical reaction is chiefly in the secondary syn clefts between the nerve cell muscle cell Bar indicates 1 μ

decide how close to the recording point itself he was working This speeded work up considerably The marking method did not hamper the histochemi staining of the myoneural junction by the method described by Joo Savay and Csi (1965) This histochemical method is suitable also for electronmiscroscopic stu of the motor end plate and therefore the marking method reported is suitable a for electrophysiologic and electronmiscroscopic study of the motor end plate at t cellular level Fig 7 is an electronmicroscopic picture of a myoneural junctio stained histochemically by the method of Joo Savay and Csillik (1965)

The present method is useful in physiologic research and especially for patholog studies in which it is important to be able to compare by means of functional change the anatomic changes that have taken place in the muscle cell of myoneural junc tion Other marking methods that may be suitable for muscle cell study are th macromolecular antibodies or bacteria fixed to the microelectrode which were sug gested by Frank and Becker (1964 A tempting possibility may exist in the sphere of autoradiography

Financial support has been gratefully received from the Pehr Oscar Klingendall Foundation and the University of Helsinki

References

BULTITUDE K H A technique for marking the site of recording with capillary micro-electrodes *Quart J micr Sci* 1958 *99* 61—63

FRANK K and M C BECKER Microelect des for recording and stimulation *In* NASTUK W L *Physical techniques in biolog cal r arch* New York and London Academic Press 1964 pp 30—87

GALIFRET Y and TH SZABO Locating cap l ary microelectrode tips within nervous t sue *Nature* (Lond) 1960 *188* 1033—1034

GROSSMAN R G L WHITESIDE and T L HAMPTON The time course of evoked depolariza tion of cortical glial cells *Brain Res* 1969 *14* 401—415

HUBEL D H Single unit activity in striate cortex of unrestrained cats *J Physiol* (Lond) 1959 *147* 226—238
HYVARINEN J, Analysis of spontaneous spike potential activity in developing rabbit diencephalon *Acta physiol scand* 1966 *68* Suppl 278
JOO E G SAVAY and B CSILLIK A new modification of the Koelle Friedenwald method for the histochemical demonstration of cholinesterase activity *Acta histochem* (Jena) 1965 *2?* 25—40
KATZ B and S THESLEFF On the factors which determine the amplitude of the miniature end plate potential *J Physiol* (Lond) 1957 *137* 267—278
KATZ B and R MILEDI Propagation of electric activity in motor nerve terminals *Proc roy Soc Edinb B* 1964 *161* 453—482
KRNJEVIĆ K and R MILEDI Failure of neuromuscular propagation in rats *J Physiol* (Lond) 1958 *140* 440—461
LEE B B G MANDL and J P B STEAN Micro-electrode tip position marking in nervous tissues a new dye method *Electroenceph clin Neurophysiol* 1969 *27* 610—613
MACNICHOL JR E J and G SVAETICHIN Electric responses from the isolated retinas of fishes *Amer J Opthal* 1958 *46* 26—46
N ... illary microelectrodes and ... muscle cell *Scand J clin*
N ... he muscle cell on electron ... 108
O ... cone action potential *J*
... cal neurones responding with ... 104
... iedeberg s *Arch exp Path*
REVEL J P ... studies of inter cellular jur
ROBINSON B ... *6* 201—223
ROSENFALCK ... nd muscle fibres A physico- ... 1969 Suppl 321
SAVAY G a ... *Nature* (Lond) 1958 *181*
SCH ...
So
ST
ST
ST
THOMAS R C and V J WILSON Precise localization of Renshaw cells with a new marking technique *Nature* (Lond) 1965 *206* 211—213
THOMAS R C and V J WILSON Marking single neurons by staining with intracellular recording microelectrodes *Science* 1966 *151* 1538—1539
USSING H H and E E WINDHAGER Active sodium transport at the cellular level *In* DE GRAFF I and B LEIJNSE Water and Electrolyte Metabolism Amsterdam Elsevier 1964 pp 3—19
VILLEGAS L Cellular location of the electrical potential difference in frog gastric mucosa *Biochem biophys Acta* (Amst) 1962 *64* 359—367

Acta physiol. scand. 1970. 80. 370—377
From the Department of Physiology, University of Helsinki Finland

Correlation between the Rise Time of Miniature End-Plate Potential and the Distance of the Recording Microelectrode from the Myoneural Junction

By

J NICKELS

Received 16 March 1970

Abstract

NICKELS, J *Correlation between the rise time of miniature end-plate potential and the distance of the recording microelectrode from the myoneural junction* Acta physiol scand 1970 *80* 370—377

A new marking method which permits accurate positioning of the microelectrode tip in the muscle cell and subsequent histochemical staining of the myoneural junction was used to study the correlation between the rise time of miniature end plate potentials and the distance of the recording microelectrode from the myoneural junction The muscle examined was the tibialis anterior of the rat. The recording was performed in vivo A positive correlation was established between the distance and the rise time and the correlation coefficient was +0 94 The mean rise time of the miniature end plate potentials in different muscle cells ranged from 0 27 to 0 70 msec while the distance of the recording microelectrode varied between 0 and 552 μ measured from the midpoint of the motor end plate The rise time was distinctly shorter than is usually reported in the literature

Acetylcholine is liberated spontaneously from the myoneural junction This process causes small depolarizations at the junction (miniature end-plate potentials mepp) The first to report this phenomenon were Fatt and Katz in 1950 It soon appeared that the rise time of these potentials varied with the distance from the myoneural junction of the recording in accordance with the volume conductor theory This discovery was used in trying to get as close as possible to the end plate in which the rise time of mepp was shortest Katz and Thesleff 1957) In addition Katz and Thesleff controlled the positioning of the microelectrode by following the fine nerve branches visible in preparations However it is not possible accurately to determine the final location with this method

The purpose of the present work was to make a more thorough study of the correlation between the rise time of mepp and the distance of the microelectrode from the myoneural junction by using a method (Nickels 1968) by which the location of the electrode tip in the muscle cell can be accurately determined Attention was moreover paid to the fairly varying results reported in the litterature for the rise time

Material and methods

White rats weighing from 130 to 200 g were the experimental animals They were anesthetised with 40—50 mg/kg of i p administered Nembutal® The animals were pinned to a cork board The m tibialis anterior was dissected free The muscle was moistened constantly with Ringer's mammalian solution of room temperature The rats were never warmed artifically during the experiment The surface muscle temperature was 28° ± 2° C The motor end plate zone itself was found fairly easily in the m tibialis anterior, approximately in the middle of the muscle

Glass capillary microelectrodes filled with 1 1 M of $K_4Fe(CN)_6$ and 1 5 M of Na_2HPO_4 were used in the recording procedure The tip of the electrodes was about 0 5 μ thick and their resistance was 5—10 MΩ The miniature end plate potentials were taken on a tape recorder with a frequency response of 40—16,000 Hz The potentials were later exposed on … were … micro- … m each … (0 5—

The location of the microelectrode tip was marked immediately after the recording by driving the anions from the electrode into the muscle cell using DC of about 2 μA The …

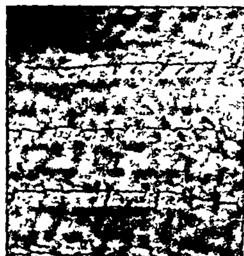
… myoneural junction was situated on the superior surface of the muscle cell it was visualised 5—10 min after incubation Mostly, however, it was on the interior surface and did not emerge readily even after prolonged incubation For this reason, individual cell preparation was performed and the myoneural junction stained easily The necessary measurements under the microscope were made immediately after this because the glycerine-gelatin mixture in which the preparation was kept under a cover glass caused some shrinkage of the muscle cell The distance between the midpoint of the sediment and that of the motor end plate was taken as the distance to be measured The sediment was between 5 and 20 μ thick Because the micro- …

where there was … other fixed point

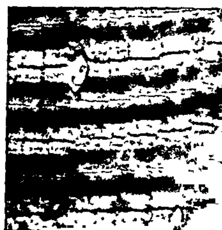
Some muscle cells were also examined electronmicroscopically to ascertain the contractive influence of electric current in the region of the sediment As the sediment contains heavy metals it is visualised electronmicroscopically For this reason the muscle cell was fixed for another hour in phosphate buffered (pH 7 4) 5 per cent glutaraldehyde, and then given the routine electronmicroscopic treatment

Results

The microelectrode tip was successfully marked in about 50 per cent of the cases The usual reason for failure was inability to dissect free the superficial fasciae Otherwise the lead reacted with the connective tissue and did not affect the region around the microelectrode itself It was difficult to demonstrate cholinesterase if the myoneural junction was underneath the muscle cell as the acting substances were not able to diffuse from between the muscle cells This required a fairly long incubation period for which a single cell preparation was usually made, and then the



a



b

Fig 1 a Electronmicroscopic view of the location of the marking sediment inside the m cell ×7500
1 b Electronmicroscopic view of the control point in the same muscle cell ×7500

incubation period was 5—10 min As the muscle was fixed in situ the state of traction corresponded to the resting length of the muscle cell Fixation was c menced before the electrophoresis current was switched on Consequently the cur caused no great contraction in the muscle cell (Fig 3) The electronmicrosc picture of the region of the sediment (Fig 1) shows a shrinkage of the sarcomer about 15 per cent compared with the control area This shrinkage causes a shorte of approx 3—4 μ in the muscle cell over a distance of 20 μ This shrinkage

TABLE I

Distance of the microelectrode from the midpoint of the myoneural junction (μ)	Mean of the rise time of 50 successi mepp (SD in brackets)	
0	0 27	(0 07)
35	0 27	(0 07
40	0 29	(0 06)
48	0 31	(0 09
52	0 27	(0 10
52	0 30	(0 10
55	0 29	(0 10)
65	0 38	(0 12)
130	0 29	(0 07)
163	0 31	(0 10)
177	0 43	(0 12)
253	0 52	(0 14)
325	0 49	(0 13)
397	0 56	(0 12)
436	0 58	(0 15)
552	0 70	(0 19)

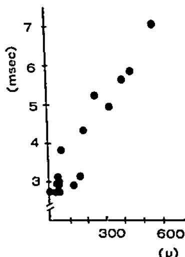


Fig 2 Relation between the distance (abscissa) of the recording microelectrode and the rise time (ordinate) of mepp Each filled circle represents the mean of the rise time of 50 successive mepp

considered to be of the same magnitude in every muscle cell No correction was made in the values stated The length of the myoneural junctions varied from 25 to 68 μ, mean 42 μ

Sixteen successful recordings and markings were made The distance of the recording site from the midpoint of the myoneural junction was 0—552 μ (Table I) The mean of the mepp rise times of the motor end plates varied from 0 27 to 0 70 msec (Table I) the shortest mepp lasted about 0 1 and the longest 1 2 msec. The correlation between the rise time and the recording distance (Fig 2) was positive, coefficient +0 94 (Fig 3 and 4) When the distance from the myoneural junction was longer the variation in rise times increased (Table I and Fig 5) As the lower frequency response of the tape recorder was 40 Hz distortion of the slower descend ing part of mepp occurred which explains why the decay time was not measured Miniature potentials were difficult to distinguish from the background noise level when the distance from the myoneural junction was over 500 μ

Discussion

The experimental arrangement gave a distinctly positive correlation between the recording distance and the rise time of mepp This concurs with previously reported experimental results (Katz and Thesleff 1957) though they did not attempt a more accurate determination of the relation between the recording distance and the rise time A point that attracts attention in the present study is the shortness of the mepp rise time It was distinctly shorter than the measurements reported in the literature Fatt and Katz (1952) stated the rise time of mepp in the frog muscle to be about 1—2 msec They gave the rise time of extracellularly recorded miniature potentials as one fifth of the value obtained by intracellular recording Later, Boyd and Martin



Fig 3 The myoneural junction (white arrow) and the microelectrode recording site marked with sediment (black arrow). The recording distance from the midpoint of the myoneural junction in Fig a is 0 μ in Fig b 130 μ in Fig c 325 μ and in Fig d 436 μ. The bar indicates 20 μ

(1956) reported a rise time of somewhat under 1 msec at 37° C in the m tenuissimus of cat. It was prolonged to about 2 msec at 20 C. Mammalian muscle thus appeared to differ from frog muscle in this respect. On the other hand Liley (1956) reported that the rise time in rat muscle at 37 C was 1 35 ± 0 28 SD msec in an intracellular and 0 80 ± 0 2 msec in an extracellular recording. In 1962 Diamond and Miledi

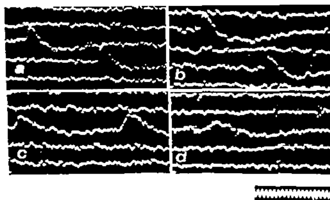


Fig 4 Miniature end plate potentials recorded from the muscle cells in Fig 3 Calibration 5000 c/s and 1 mV

studied phrenic nerve preparations of the rat fetus and found that its mepp rise time varying between 2 and 10 msec differed clearly from the rise time of adult rats Hubbard and Smith (1963) also studied phrenic nerve preparation from rat and reported for both extra and intracellular recordings distinctly shorter rise time of mepp than had been published earlier The former was 0 42 ± 0 06 (mean ± 1SD) and the latter 0 70 ± 0 07 msec

In 1968 Gage and Armstrong published a report of miniature end plate current (mepc) studied by the voltage clamp technique employed by Takeuchi and Takeuchi

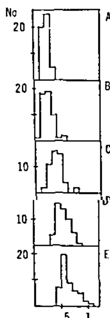


Fig 5 A histogram which illustrates the rise time of 50 successive mepp Distance of the microelectrode from myoneural junction a) 0 μ b) 52 μ c) 1 7 μ d) 3?5 μ e) 436 μ Ordinate numbers of observation abscissa rise time of mepp

(1959) for the study of end plate potental (epp) The method can be used to measure the active phase of the potential which also illustrates the current that originates Gage and Armstrong reported that the time interval to the mepc peak was 150—300 μs The respective rise time of mepp would be correspondingly shorter than earlier results indicate The same authors obtained the same results for frog muscle In all the experiments mentioned the aim was to make the mepp recordings as close to the myoneural junction as possible, and they are thus comparable with my recordings at a distance of under 60 μ These would seem to substantiate fairly well the results arrived at by Gage and Armstrong Inconsistencies with earlier results as far as the myoneural junction of the frog is concerned, can be accounted for partly by the wider spread of the synaptic region in the frog than in the myoneural junction of mammals (Kuhne 1887) The slow rise times of fetal muscle my be due to the prolonged action of acetylcholine, for the amount of cholinesterase at the myoneural junction is smaller than in an adult An additional factor, according to Diamond and Miledi, may be various electrical properties of the muscle cell membrane Boyd and Martin (1956 b) compared the 0 6 msec rise time of mcpp with the corresponding 0 8 msec rise time for mepp and assumed that the shorter rise time was due to the more inaccurate localisation of the electrode tip in measuring mepp because the myoneural junction is easier to localise with the help of the great amplitude and typical form of mepp than by means of the shape of mepp However my study suggests that the distance may vary considerably without definite differences arising in the rise time of mepp All considered it is thus fairly difficult to explain the great variation in the rise times previously established

I heated neither the muscle itself nor Ringer s solution because it is fairly difficult to standardise this when the moisturising layer is fairly thin and the increased vaporisation from heating with infrared rays adds to the unevenness of the temperature Nor was it possible to use in vivo recording the muscle covered with mineral oil employed by Roberts and Thesleff (1965) as it would have interfered with the post recording marking The experiment was therefore conducted at room temperature with Ringer s mammalian solution of room temperature The temperature of the superficial muscle cells was thus lower than normal A low temperature is known to prolong the rise time of mepp Boyd and Martin (1956 a) observed that the rise time of mepp was prolonged in the m tenuissimus of cat from under 1 msec to 2 msec when the temperature dropped from 37 to 20 C According to Liley (1956) a corresponding change in temperature doubled the rise time in the rat muscle cell This makes it possible that the rise times established in the present study might be even shorter However the myoneural junction was almost regularly on the interior surface or in the side of the muscle cell and the end plate temperature was thus probably fairly close to normal

The fairly great variation in the rise time of mepp even in the same muscle cell when recording is d[illegible] at the same distance has been noted earlier (Del Castillo and Katz 1956) This ar[illegible] in favour of the assumption that there are great reactive variations at the my[illegible]ural junction obviously between Ach and the receptor

molecules The same fluctuation in the rise time was observed in the present study (Fig 5) It increased with the distance This must surely be due in part to the diminution of the amplitude of mepp when distance increases causing an increase in measuring errors

The relatively great variation of the mean rise times in recordings made at less than 100 μ distance also attracts attention It is possible that the variations in the membrane characteristics of the muscle cells, temperature variations and measuring errors are the factors in question here Katz and Thesleff (1957) stated that the rise time of mepp was slightly shorter in thinner muscle cells In this work, however, it was impossible to consider the calibre variation of the muscle cells as it would have necessitated making a cross section of the muscle cell It must also be recognised that the m tibialis anterior of rat contains both so-called slow (red) and fast (white) muscle cells and their membrane qualities may differ

This study has been aided by grants from the Pehr Oscar Klingendahl Foundation and the University of Helsinki

References

BOYD I A and A R MARTIN Spontaneous subthreshold activity at mammalian neuromuscular junctions *J Physiol* (Lond) 1956 a *132* 61—73

BOYD I A and A R MARTIN The end plate potential in mammalian muscle *J Physiol* (Lond) 1956 b *132* 74—91

CASTILLO J DEL and B KATZ Localization of active spots within the neuromuscular junction of the frog *J Physiol* (Lond) 1956 *132* 630—649

DIAMOND J and R MILEDI A study of foetal and new born rat muscle fibres *J Physiol* (Lond) 1962 *16?* 393—408

FATT P and B KATZ Some observations on biological noise *Nature* (Lond) 1950 *166* 597—598

FATT P [illegible] at motor nerve endings *J Physiol* Lonc [illegible]

GAGE F [illegible] currents in voltage-clamped muscle fibre [illegible]

HUBBARD J I and R F SCHMIDT An electrophysiological investigation of mammalian motor nerve terminals *J Physiol* (Lond) 1963 *166* 145—167

JOO F G SAVAY and B CSILLIK A new modification of the Koelle Friedenwald method for the histochemical demonstration of cholinesterase activity *Acta histochem* (Jena) 1965 *22* 40—45

KATZ B and S THESLEFF On the factors which determine the amplitude of the miniature end plate potential *J Physiol* (Lond) 1957 *137* 267—278

KUHNE W Neue Untersuchungen uber motorische Nervenendigung *Z Biol* 1887 *23* 1—148

Li [illegible] ular junction of the rat

N [illegible] muscle cell on electron

R [illegible] no and the actions of decamethonium a micro-electrode study *Acta anaesth scand* 1965 *9* 165—172

TAKEUCHI A and N TAKEUCHI Active phase of frog s end plate potential *J Neurophysiol* 1959 *22* 395—411

Acta physiol. scand. 1970. 80. 378—382

From the Institute of Neurophysiology, University of Copenhagen Denmark

Contraction Times of Twitches Evoked by H-Reflexes

By

Fritz Buchthal and Henning Schmalbruch[1]

Received 20 March 1970

Abstract

Buchthal F and H Schmalbruch *Contraction times of twitches evoked by H reflexes* Acta physiol scand 1970 *80* 378—382

Isometric twitches of the same amplitude were initiated in the human soleus muscle by near threshold H reflexes and by stimuli to the efferent nerve (M responses) or to the end plate zone The time to peak force was longer in reflex than in directly activated twitches The range was 84 to 130 msec and 52 to 100 msec respectively 36—37° C)

In cat tonic motoneurones activated by a stretch reflex have smaller axons than phasic motoneurones (Granit Henatsch and Steg 1956, Henneman Somjen and Carpenter 1965 they have a lower threshold conduct slower and innervate muscle fibres with slow contraction times (Eccles Eccles and Lundberg 1958, Henneman and Olsen 1965 Similarly in human muscles of the calf the contraction times were longer when elicited by the H-reflex than when elicited through the efferent nerve or by the myotatic refex Homma and Kano 1962

In a previous study we recorded mechanical responses of small bundles of fibres in different human muscles Buchthal and Schmalbruch 1970 In the study reported here discrete twitches in the soleus muscle were elicited by direct electrical stimuli in the end plate zone and through the H reflex The aim was to establish whether the fibres activated were the same or whether the reflexly activated fibres were slow as in the cat

Method

1 Stimulus

Reflex responses H and direct responses M) were evoked in the soleus muscle by stimulating the tibial nerve in the popliteal fossa Fig 1 The stimulus was a rectangular pulse 0 2 msec in duration applied via a double screened transformer through needle electrodes or through surface electrodes The site of lowest threshold for the reflex response (H) was located by small displacements of the stimulating cathode The rate of stimulation was less than 0 5/sec When the rate was increased t 1 2 sec only every fifth or tenth stimulus evoked a reflex response and it was possible [illegible] late the M response on which the reflex twitch was superimposed.

The reflex resp[illegible] H was also compared with twitches of the same amplitude evoked

[1] Working under a [illegible] ship from the Rask Ørsted Foundation Copenhagen and the Fritz Poensgen-Stiftung Du[illegible]dorf on leave from the Institute of Biophysics and Electronmicroscopy University of Dussel

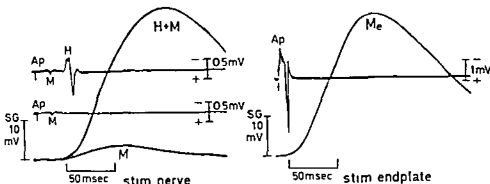


Fig 1

Left H+M twitch response of the soleus muscle evoked by an H reflex superimposed on a small M response

The lower twitch is the M response isolated by increasing the rate of the stimuli Ap Action potentials of the M and of the H responses

Right M_e response evoked by a stimulus in the end plate zone

S G Output of the strain gauge in mV

Subject I M male 19 years old

The intramuscular temperature was 37° C

directly by stimuli in the end plate zone of the soleus muscle (M_e) Two needle electrodes 5 cm apart were inserted from the lateral surface of the leg anterior to the tendon of the gastrocnemius muscle *The stimuli were adjusted in strength so twitches activated reflexly and* from the end plate zone were equal in amplitude (Fig 1)

2 *Recording of twitch responses*

The subject was in a prone position with the leg on a splint the calf muscles slightly stretched and the ankle joint fixed at 90° by a support beneath the sole The method of recording discrete mechanical responses from a muscle *in situ* has been described Buchthal and Schmal [illegible]

3 *Recording of action potentials*

Action potentials were recorded from the soleus and the lateral and medial heads of the gastrocnemius muscle (i) to identify the presence of a reflex response (ii) to determine the onset of contraction in an H reflex contaminated by the preceding M response (see next section and iii) to ascertain that the mechanical responses originated from the soleus muscle alone To lead-off from the soleus muscle surface electrodes (chlorided silver discs 5 mm in diameter filled with electrode paste were attached above the belly and the lateral malleolus To lead-off from the lateral and medial heads of the gastrocnemius muscle concentric electrodes were used

The output of the transducer and the action potentials were displayed on the beams of a storage oscilloscope (Tektronix 564 time base 200 or 500 msec) and photographed with a Polaroid camera

4 *Measurement of the time of peak of the twitch*

Contraction times are usually g [illegible]
to the peak force The onset [illegible]
rendering the initial deflectior [illegible]
the negative peak of the actior [illegible]

contraction. The electrode on the belly of the soleus muscle was moved along the muscle until the peak of the action potential coincided with the onset of the M response. The contraction time of the H reflex was measured from the negative peak of the action potential preceding the reflex twitch to the peak of the force of the compound twitch (M + H).

5. Response to paired stimuli.
To evaluate the effect of the preceding M response on the contraction time of the H reflex two stimuli were applied to the end plate zone with a time interval of 20 msec, similar to the time between the action potentials of the M- and of the H responses. The stimuli of the pair were either delivered to the same point in the end plate zone or, since different fibre groups are activated in the M- and in the H responses, via four electrodes to adjacent points.

6. The temperature was maintained at 36 to 37° with an infrared lamp and measured throughout the experiments by a thermo-couple inserted into the muscle.

7. The experiments were performed on 6 subjects, 4 males, 2 females, 18—20 years old without signs or symptoms of neuromuscular disease.

Results

1. Comparison of H-, M- and M_e-responses

The contraction times of weak reflex responses in the soleus muscle were about 30 % longer than of M-responses ($p < 0.001$, Fig. 2). The time to crest of reflex twitches was 98 ± 2 msec ($n = 17$), of M-responses 71 ± 2 msec ($n = 10$), and of responses to stimuli in the end-plate zone (M_e) 76 ± 2 msec ($n = 17$). Near-threshold H reflexes were compared both with M- and M_e-responses of the same amplitude. Stronger H-responses were compared only with twitches evoked by stimuli to the end-plate zone since stimuli to the nerve strong enough to elicit twitches of the same amplitude also activated the gastrocnemius muscle.

The contraction time decreased slightly with increasing force of the H reflex ($p <$

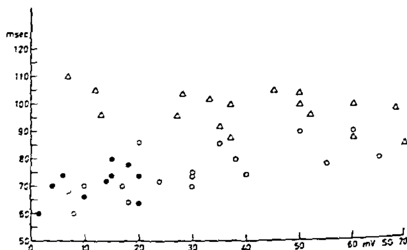


Fig. 2. The contraction times (ordinate) of twitches of different amplitude (abscissa) in the soleus muscle evoked by H reflexes, of M responses and of M_e responses to stimuli in the end plate zone. Two normal subjects, intramuscular temperature 37° C.

△ H reflexes
● M responses: stimuli to the efferent nerve
○ M_e-responses, stimuli in the end plate zone

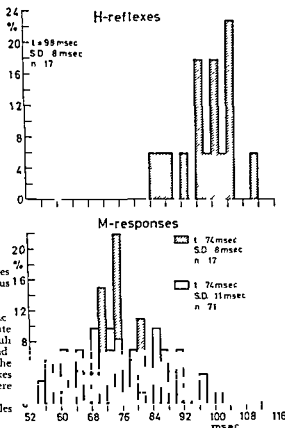


Fig 3 Histograms of contraction times (t) of twitches in the human soleus muscle (36—37° C)
Above Evoked by H reflexes
Below Evoked in small bundles activated by stimuli to the end plate zone (white columns) and by stimuli to the efferent nerve and to the end plate zone (striped columns) in the same subjects in whom the H reflexes shown in the upper histogram were recorded
n denotes the number of fibre bundles examined

0 05) and conversely, the contraction time of M_e responses increased with increasing force (p<0 001) (Fig 2)

The spectrum of contraction times was obtained by activating 15—20 small bundles in each of 6 soleus muscles The spectrum of reflex twitches was confined to the portion of the spectrum with long contraction times (Fig 3)

2 Effect of the preceding M response on the H response

In model experiments with paired stimuli 20 msec apart in the end plate zone the time from the second stimulus to peak force was the same whether it was preceded by another twitch or not Even when the two twitches were of equal amplitude and when the two stimuli were applied to the same point or to adjacent points in the end plate zone the response to paired stimuli had the same time to peak as the response to one stimulus In fact when M and H responses were paired the M response had an amplitude only 10—20 percent of the reflex twitch

Discussion

The experiments on the soleus muscle of human subjects have demonstrated only the slower fibres within the muscle contribute to the reflex twitch Th

contraction. The electrode on the belly of the soleus muscle was moved along the muscle until the peak of the action potential coincided with the onset of the M response. The contraction time of the H-reflex was measured from the negative peak of the action potential preceding the reflex twitch to the peak of the force of the compound twitch (M+H).

5. Response to paired stimuli

To evaluate the effect of the preceding M-response on the contraction time of the H reflex two stimuli were applied to the end-plate zone with a time interval of 20 msec, similar to the time between the action potentials of the M- and of the H-responses. The stimuli of the pair were either delivered to the same point in the end-plate zone or, since different fibre groups are activated in the M- and in the H-responses, via four electrodes to adjacent points.

6. *The temperature* was maintained at 36 to 37° with an infrared lamp and measured throughout the experiments by a thermo-couple inserted into the muscle.

7. *The experiments were performed on 6 subjects*, 4 males, 2 females, 18—20 years old, without signs or symptoms of neuromuscular disease.

Results

1. Comparison of H-, M- and M_e-responses

The contraction times of weak reflex responses in the soleus muscle were about 30 % longer than of M-responses ($p < 0.001$, Fig. 2). The time to crest of reflex twitches was 98 ± 2 msec ($n = 17$), of M-responses 71 ± 2 msec ($n = 10$), and of responses to stimuli in the end-plate zone (M_e) 76 ± 2 msec ($n = 17$). Near-threshold H-reflexes were compared both with M- and M_e-responses of the same amplitude. Stronger H responses were compared only with twitches evoked by stimuli to the end-plate zone since stimuli to the nerve strong enough to elicit twitches of the same amplitude also activated the gastrocnemius muscle.

The contraction time decreased slightly with increasing force of the H reflex ($p <$

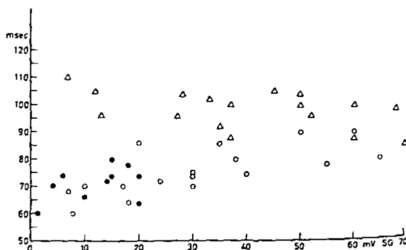


Fig. 2. The contraction times (ordinate) of twitches of different amplitude (abscissa) in the soleus muscle evoked by H reflexes, of M-responses and of M_e responses to stimuli in the end plate zone. Two normal subjects, intramuscular temperature 37° C.

△ H-reflexes
● M-responses, stimuli to the efferent nerve
○ M_e-responses, stimuli in the end-plate zone

Acta physiol. scand. 1970. 80. 383—394

From the Department of Physiology, University of Goteborg Sweden

Circulatory Effects of Interruption and Stimulation of Cardiac Vagal Afferents

By

B Öberg and S White[1]

Received 23 March 1970

Abstract

Öberg, B and S White *Circulatory effects of interruption and stimulation of cardiac vagal afferents* Acta physiol scand 1970 *80* 383—394

[illegible] produced a blood [illegible]

The presence of receptors in the cardiac chambers is well established [illegible] logical studies and electrophysiological recordings of the rhythmic [illegible] afferents from the heart (Amann and Schaefer 1943, Jarisch and [illegible] Whitteridge 1953, Paintal 1953 1955) The functional role of the [illegible] in circulatory control remains however, largely unknown, partly [illegible] tive inaccessability of the receptor sites and partly because the [illegible] nature of their reflex effects are probably disturbed by the [illegible] necessary for adequate study Furthermore, knowledge with [illegible] stimulus for the different types of receptors is still limited, [illegible] atrial receptors type B, the adequate stimulus appears related to the [illegible] of atrial muscle fibres (Paintal 1963)

On the occasions when cardiac receptors have been stimulated by [illegible]

[1] Overseas Research Fellow of The Life Insurance Medical Research [illegible] New Zealand *Present address* Hallstrom Institute of Cardiology, [illegible] Missenden Rd Comperdown 2050 N S W, AUSTRALIA

mechanical means or afferents from the receptors stimulated electrically, depressor responses manifest in hypotension bradycardia and peripheral vasodilatation have been usually observed (Bezold and Hirt 1867, Daly and Verney 1926 Jarisch and Zotterman 1948, Aviado *et al* 1951, Salisbury, Cross and Rieben 1960, Ross Frahm and Braunwald 1961) The details of this response patterns however, remain to be examined

Based on relatively few pertinent data in this field several hypotheses have been proposed concerning the functional role of cardiac receptors in the control of the circulation It has for example, been suggested that they play a role in volume regulation (Gauer and Henry 1963) in the 'vaso vagal syncope' following haemorrhage (Pearce and Henry 1955) and in pathophysiological states such as myocardial infarction (Kolatat *et al* 1967) According to Neil (1962) they may also exert a particularly strong influence on the venous side of the circulation and thereby constitute part of an effective feedback system directed to the maintenance of central venous pressure

The present series of experiments were undertaken to examine in detail reflex circulatory adjustments that occur through cardiac vagal nerves in the cat known to be composed mainly of sensory afferents from the heart (Jarisch and Zotterman 1948 Paintal 1964) The circulatory effects of both interruption and afferent electrical stimulation of the cut cardiac nerves were observed and the effects consistently compared in the same animal with those obtained by the 'unloading or stimulation of the carotid sinus baroreceptors to permit an approximate quantitative estimate of the relative power of the two afferent reflex mechanisms under study

Methods

Experiments were performed on 37 cats anesthetized with chloralose 30 50 mg/kg b w A tracheal cannula was inserted and the carotid arteries and vagi on both sides dissected free in the neck and separated from each other for a long distance The aortic nerves were identified and cut as they joined the superior laryngeal nerves

When the cardiac nerves were used in the experiment thoracotomy was performed through right and left intercostal incisions with the animal on positive pressure ventilation The nerves were dissected free as close to the heart as possible and placed on loose ligatures for later section or electrical stimulation For section the constant right cardiac nerve and the multiple cardiac branches that leave the main trunk of the left vagus immediately caudal to the appearance of the recurrent laryngeal nerve were included in the ligatures For electrical stimulation *the vena azygos was divided and the right cardiac nerve* was dissected free and cut as close to the heart as possible before mounting on stimulating electrodes Systemic arterial pressure (subclavian artery and in some experiments right atrial pressure (via a catheter passed through the external jugular vein were measured using Statham P23AC and P23BC strain gauges respectively Heart rate was monitored by a tachograph triggered by the rapid upstroke of the arterial pressure pulse Blood flow from the calf muscles the kidney and a part of the small intestine was measured by cannulating the respective draining vein and passing the blood through an optical drop recorder operating an ordinate writer Recordings of the above circulatory variables were made on a Grass polygraph recorder Alterations in venous tone in skeletal muscle and intest ne [illegible] e volume recorded with a plethysmographic [illegible] 1960 Follow [illegible] *al* 1963 Alterations in flo [illegible] calculated from perfusion pressure and bloo [illegible] riments perfusion pressure was maintained constant during the various procedures either by connecting the arterial side of the circulation via the femoral or mesenteric artery to a simple pressure compensator or by perfusing the vascular beds under study from a donor animal

was taken to ensure nearby vagal structures were not disturbed by this procedure

Heparin, 5 mg/kg was used as anticoagulant. In some experiments atropine, 0.4 mg/kg was given intravenously to effect cardiac parasympathetic blockade

Results

1 Circulatory responses to interruption of cardiac vagal afferents, following section of the aortic nerves

Circulatory responses to cervical vagal cooling were systematically analyzed and compared with the effects of bilateral carotid occlusion in 13 animals In 5 of the experiments, atropine was given prior to vagal cooling A record from one representative experiment is shown in Fig 1, while mean data from all experiments are presented in Table I

Cooling of the cervical vagi (C, Fig 1, column I, Table I) regularly produced a marked augmentation in heart rate (in non atropinized animals at least partly due to cold block of efferent fibres), an increase of both muscle and renal flow resistance, and a slight fall in right atrial pressure Arterial pressure usually rose somewhat but was virtually unchanged in three animals The circulation was "normalized" to control values on rewarming of the vagi, often after a shortlasting 'overshoot' The results suggest that afferent impulses normally travel in the cervical vagi to place an inhibitory restrain on central vasomotor neurons

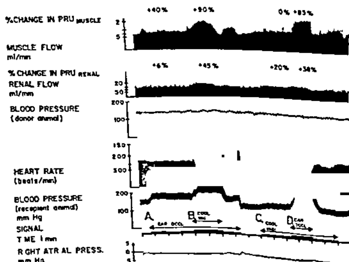


Fig 1 Recipient animal 2.2 kg Chloralose 50 mg/kg body weight. Both aortic nerves cut in the neck

Effects of carotid occlusion (A), cervical vagal cooling (C) and superimpositions of the two procedures (B and D) on arterial blood pressure right atrial pressure, heart rate and skeletal muscle and renal blood flows The vascular beds were perfused with constant pressure from donor animal

T a b l e

	I Cooling of vag	II Bilateral carot d occlus on	III Cool ng of vagi dur ng per od of b lat carot occl		IV Cool ng of vagi after atrop ne Both carot ds occluded In crease from pre cool control
			increase from pre cool con trol	increase from pre occlus on control	
Heart rate	7±2 4*	1 5±1 9	5±1 0*	14±4 4*	4 5±1 2*
Blood pressure	3±2 5	29±7 6*	19±6 0*	47±10*	16±4 2*
Muscle PRU	10±3 0*	53±7 5*	24±5 8*	78±12*	25±5 3*
Renal PRU	16±4 3*	19±5 4*	37±10*	60±20*	20±4 6*

The vagally mediated inhibitory influence was however when evaluated in general terms *e g* blood pressure responses far less powerful than that exerted by the arterial baroreceptors at least as long as the latter receptors were allowed to display their buffering influence undisturbed (Compare A and C Fig 1 and column I and II Table I)

Comparisons of the circulatory responses to vagal cooling and reduced baroreceptor activity (carotid occlusion) also revealed qualitative differences with respect to the degree of engagement of the various cardiovascular target organs Carotid occlusion thus regularly produced much more pronounced vascular responses in the skeletal muscle than in the kidney and practically no changes in heart rate while vagal cooling consistently led to a more powerful renal vessel constriction and marked increases in heart rate The tachycardia of vagal cooling although somewhat reduced in extent also appeared in atropinized animals (Table I column IV) These findings suggest that inhibitory impulses travelling in vagal afferents converge mainly on central vasomotor neurons controlling the efferent discharge to the renal vessels and to the heart In contrast baroreceptor afferents seem to be preferentially orientated to skeletal muscle neurons and to a less extent to central neurons affecting the renal vessels and the heart

While the circulatory responses to vagal cooling were moderate in the presence of *normally functioning baroreceptors* they became much more pronounced when the baroreceptor buffering mechanisms were disturbed prior to the cooling by *e g* carotid occlusion (B in Fig 1 column III in Table I) Interruption of vagal afferent impulses then induced a marked increase in blood pressure and flow resistances in both vascular beds despite an initial high background vasoconstrictor fibre discharge produced by carotid occlusion The responses observed when vagal cooling and carotid occlusion were combined generally exceeded to a marked extent those expected to occur by a mere summation of the responses to each *individual intervention* This phenomenon was also evident when carotid occlusion was superimposed on vagal cooling (D in Fig 1) In the absence of the normal vagal afferent in

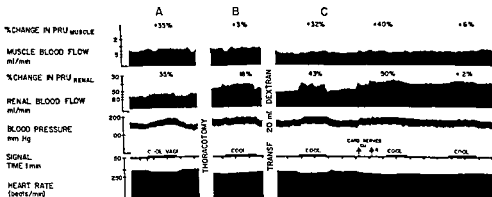


Fig 2 Cat 3 4 kg Chloralose 35 mg/kg body weight. The animal was atropinized (0 5 mg/kg)

hibitory activity, carotid occlusion can be seen to produce a marked tachycardia in contrast to the case when the vagi were intact This finding seems to support further the idea of an inhibitory influence of vagal afferents on central cardioaccelerator neurons

Cooling of the cervical vagi also results in blockade of afferent impulses from extracardiac receptors in the lungs viscera etc To test the contribution of afferents outside the cardiac nerves to the inhibitory activity present in the cervical vagi, a series of experiments were performed on 4 cats where the effects of vagal cooling were compared before and after section of the cardiac nerves Records of one such experiment on an atropinized cat with both carotids occluded are shown in Fig 2, and the results from all 4 experiments are collected in Table 2 Cervical vagal cooling (A Fig 2) produced the type of response described above *i e* a rise in blood pressure vasoconstriction in renal and skeletal muscular beds and increased heart rate despite atropine To gain access to the cardiac nerves close to the heart, the thorax was opened and positive pressure breathing instituted These procedures in themselves caused a slight increase in heart rate and regional flow resistance (B Fig 2), and the circulatory responses to vagal cooling became somewhat attenuated These phenomena might be explained partly by an unloading of heart receptors and diminution of vagal afferent activity, consequent upon shrinkage of the heart when the chest was opened After expansion of the circulatory system by dextran transfusions the responses to vagal cooling recovered (C in Fig 2) The response to cervical vagal cooling was then closely mimicked both quantitatively and qualitatively by cutting the cardiac nerves close to the heart Cold block of vagi after sectioning the cardiac nerves resulted in only trifling increases in heart rate, arterial pressure and regional flow resistance The results indicate that the major part of the tonic vagal afferent

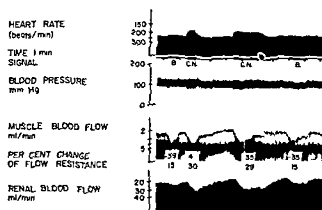


Fig 3 Cat 3 1 kg Chloralose 40 mg/kg body weight
Effects of mechanical stimulation of the carotid sinus baroreceptors (B) and afferent cardiac nerve stimulation (CN) (4 imp/sec, 8 volts, 1 msec) on heart rate and muscle and renal blood flows Arterial blood pressure maintained constant throughout — Note that for equal reductions in skeletal muscle flow resistance with the two reflex mechanisms cardiac nerve stimulation produces more marked effects on heart rate and renal flow resistance

vical vagal cold block travels in the cardiac nerves However, some inhibitory activity evidently originates from sources other than those served by the cardiac nerves sectioned

2 Circulatory responses to stimulation of cardiac vagal afferents

The effects of afferent stimulation of the right or left cardiac nerve with low frequency stimulation (2—15 imp/sec, 1—2 msec, 1—10 V) were analyzed in 20 cats — The responses to stimulation consisted of a fall in arterial pressure, vasodilatation in skeletal muscle renal and intestinal vascular beds and a profound bradycardia The responses were completely blocked by ipsilateral cervical vagal cooling indicating their reflex nature The vasodilatation was not affected by atropinization and was refore probably entirely due to withdrawal of sympathetic vasoconstrictor tone e role of adrenal medullary hormones in such transient responses has been shown be minor The bradycardia was however, due mainly to vagal efferent excitation since it was markedly reduced after atropine was given The reflex circulatory responses to cardiac nerve stimulation were thus qualitatively similar to those obtained with arterial baroreceptor stimulation A quantitative comparison of the reflex engagement of various cardiovascular target organs in the two types of reflex response patterns revealed however clearcut differencies This is demonstrated in Fig

TABLE II Circulatory responses to cooling of cervical vagi, section of the cardiac nerves and cooling of vagi after section of cardiac nerves The data are expressed as per cent changes from control ± S E of the mean and constitute means of means from multiple tests (column I and III) in 4 animals Statistically significant changes (p<0 02) are noted with* — Both carotid arteries occluded throughout Aortic nerves cut

	I Cooling of cervical vagi	II Section of cardiac nerves	III *Cooling of vagi after cardiac nerve section*
Heart rate	4 5 ± 1 1*	3 5 ± 0 7*	1 ± 0 4
Blood pressure	17 ± 1 7*	21 ± 5 2*	4 5 ± 2 5
Muscle PRU	48 ± 8 2*	73 ± 11 2*	18 ± 6 2
Renal PRU	31 ± 6 8*	44 ± 9*	14 ± 5 1

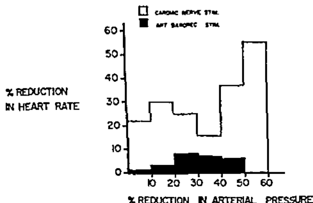


Fig 4 Relation between per cent reduction of heart rate and arterial blood pressure produced by stimulation of cardiac nerves and arterial baroreceptors The graph shows collected data from 27 stimulations of the cardiac nerves (8 V 1 msec and stimulation frequency varying between 1 and 12 imp/sec) and 22 stimulations of the carotid sinus baroreceptors in 6 animals

3 where the effects of cardiac nerve stimulation and arterial baroreceptor stimulation on heart rate skeletal muscle and renal blood flow ars shown Both types of reflex *influence can be seen to produce bradycardia and vasodilatation in both beds* However the bradycardia is much more pronounced when the cardiac nerves are stimulated Furthermore stimulation of the arterial baroreceptors produces a very small renal response compared with the effects in skeletal muscle, while with cardiac nerve stimulation the two circuits are engaged to almost the same extent These findings were consistent in all experiments and are summarized in Fig 4 and 5 In Fig 4 the per cent reduction in heart rate produced by the two types of reflex influence is plotted against the simultaneously produced per cent reduction in arterial blood pressure It can be seen that for a given arterial pressure fall there is a much more marked bradycardia with cardiac nerve stimulation than with baroreceptor stimulation In Fig 5 left panel the responses of the renal vessels are plotted against the concomitantly produced skeletal muscle vessel adjustments when the two reflexes are elicited For a given reduction in skeletal muscle flow resistance the accompanying vasodilatation in the kidney is more pronounced when cardiac nerves are stimulated than with baroreceptor stimulation Such a difference does not show up when a similar comparison between skeletal muscle and intestinal vessels is made (right panel) It therefore seems as if the inhibitory vagal afferents from cardiac receptors are more orientated towards central neurons controlling the heart and the renal vascular bed than arterial baroreceptor afferents which in turn exert a particularly *strong influence on central skeletal muscle vasomotor neurons*

Stimulation of cardiac vagal afferents regularly produced a dilatation of the veins in skeletal muscle and in the intestine together with a dilatation of the corresponding resistance vessels The magnitude of the venous responses for a given reduction in flow resistance in the two circuits was similar irrespective whether cardial vagal afferents or the arterial *baroreceptors were stimulated This is illustrated in Fig 6* where data from 3 expts on the intestinal preparation are collected The venous responses were plotted against the concomitant resistance vessel responses when cardiac nerves and arterial baroreceptors were stimulated The different poin

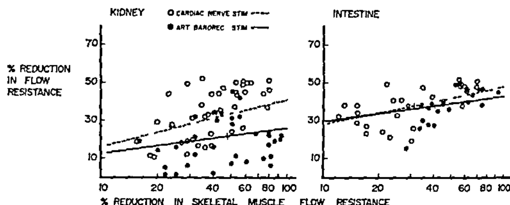


Fig 5 Per cent reduction of renal (left panel) and intestinal (right panel) flow resistance related to the simultaneously induced reduction in skeletal muscle flow resistance when cardiac nerves (open circles) and carotid sinus baroreceptors (closed circles) were stimulated Changes in skeletal muscle flow resistance have been plotted on log scale to linearize the data regression lines for cardiac nerve and arterial baroreceptor stimulation data are shown as interrupted and solid lines respectively Regression equations were *Left panel* for cardiac nerve stimulation $y = 25\ 1x - 8\ 3$ for arterial baroreceptor stimulation $y = 12\ 2x - 0\ 8$ *Right panel* for cardiac nerve stimulation $y = 20\ 1x + 8\ 1$ and for arterial baroreceptor stimulation $y = 13\ 9x + 15$ Vertical bars on each line indicates the mean Renal data from 5 cats intestinal data from 3 cats

be seen to fall practically along the same regression line — Quite similar relationship was obtained in 3 expts in the skeletal muscle (hindquarters) preparation

Clearcut responses to cardiac nerve stimulation appeared with very low stimulation frequencies usually 1—2 imp/sec With increasing frequency the responses became more pronounced reaching a maximum between 8—12 imp/sec With still higher frequencies the responses usually became reduced in magnitude Stimulation threshold was also very low usually only 1—2 V was needed to produce significant responses Maximal effects on the peripheral vascular beds were obtained with approximately 6 V while the bradycardia grew progressively more pronounced as voltage increased to 10—15 V

In 2 animals there was consistently a pressor response to cardiac nerve stimulation *i e* a constriction of the peripheral vascular beds and a blood pressure rise irrespective of the stimulation characteristics This pressor response was however always accompanied by a bradycardia A tachycardia was never seen with cardiac nerve stimulation regardless stimulation characteristics

Discussion

In the present experiments attempts were made to analyze the functional role of cardiac receptors in normal homeostatic control and to make a quantitative estimate on the relative power of the cardiac receptor system compared with arterial baroreceptors This was done by observing in one and the same animal the detailed circulatory effects of interruption of cardiac afferent and arterial baroreceptor activity or of electrical stimulation of cardiac nerves and mechanical stimulation of carotid sinus baroreceptors The approach has obvious drawbacks but can be justified as a

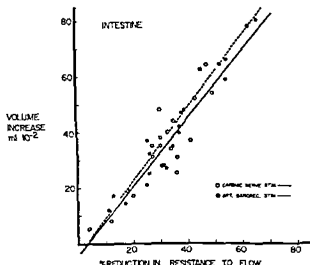


Fig 6 Relation between intestinal capacitance and resistance vessel responses expressed as per cent increase of tissue volume and reduction of flow resistance respectively to stimulation of afferent cardiac nerves (open circles) and arterial baroreceptors (closed circles) Regression lines for data from 3 cats are shown broken line for stimulation of cardiac afferents (y−1 29x −4 3) solid line for arterial baroreceptor stimulation (y−1 25x−4 7) Note that for a given reduction of flow resistance the venous responses are of equal magnitude irrespective of the type of receptor afferents stimulated

preliminary step in an analysis of circulatory control in view of technical difficulties associated with a more physiological stimulation of the receptors under examination in animals extensively prepared for studies of changes in regional blood flow and blood volume

Interruption of afferent activity in cardiac vagal fibres was carried out either by cutting the cardiac nerves in the chest close to the heart or by cooling the vagal stem at the cervical level after section of the aortic nerves The former procedure necessitated opening the chest and the application of artificial respiration with consequent disturbances in intrathoracic hemodynamics *e g* shrinkage of the heart (Rushmer *et al* 1951) This probably leads to unloading of cardiac stretch receptors and reduced activity in the afferent pathways The circulatory responses to subsequent section of the afferent pathways will then be markedly reduced or even abolished a phenomenon repeatedly observed in the present series of experiments Such undesirable effects were avoided if the thorax remained intact and cardiac afferent activity was blocked by cervical vagal cooling The simultaneous blockade of extracardiac afferents is less a problem in the analysis than appears at first sight since the circulatory response to vagal cooling was almost completely dependent on the presence of the cardiac nerves indicating that most of the vagal afferent activity inhibiting central cardiovascular neurons travels in these nerves

The results do not of course exclude the possibility that receptors located outside the heart but with their afferent pathways in the cardiac nerves contribute to the inhibitory impulse traffic There is evidence from electrophysiological recordings that at least some pulmonary inflation receptors send afferent impulses in the cardiac nerves Heymans and Neil 1958 Öberg and Thoren 1970) although the major portion of the receptors have their afferents outside the cardiac nerves (Öberg and [Tho]ren 1970) Since interruption of this influence with vagal cooling following sec[tion]

the cardiac nerves, produced relatively small effects compared to those obtained from section of cardiac nerves alone the contribution of pulmonary inflation receptor activity in the response to cardiac nerve section is probably small The role of afferents from receptors in the pulmonary vasculature in these responses is not known and a contribution from this site cannot be excluded

The results indicate that cardiac receptor nerve afferents probably exert a generalized inhibitory influence on central vasomotor neuron pools This confirms the findings by Guazzi Libretti and Zanchetti (1962) on cats and by Pillsbury, Guazzi and Freis (1969) on rabbits *This inhibitory influence is, however, as it seems not* uniform in magnitude with regard to the various cardiovascular target organs, but is preferentially orientated towards neurons controlling the activity in cardiac accelerator fibres cardiac vagal efferent fibres and renal vasomotor fibres This is in contrast to the effects of arterial baroreceptor activity, which is mainly directed to central neurons controlling the vasomotor discharge to skeletal muscle with little effect on the heart and on the renal circulation

The power of the inhibitory influence transmitted through the cardiac nerves seems to be relatively moderate and the reflex effects are to a large extent buffered by the arterial baroreceptors However when the two types of reflexes are working in consert, the contribution from cardiac receptors to the net circulatory responses is more marked The fact that the circulatory responses to simultaneous withdrawal of baroreceptor and cardiac receptor inhibitory influences are more pronounced than could be expected from a mere summation of the responses to each of the two individual reflex mechanisms suggests a certain extent of convergence of the two afferent pathways on central cardiovascular neurones

The effects of interruption of the activity in cardiac afferents must be related to cardiac receptors which are tonically active It is known that atrial receptors designated as A and B types (Paintal 1953) as well as some ventricular receptors show rhythmic discharge with each heart beat The type of receptor responsible for the *tonic inhibitory activity demonstrated in the present study cannot be clarified from* the present data

It was assumed that low frequency afferent stimulation of the peripherally cut cardiac nerves would tend to normalize the circulation *i e* bring the heart rate blood pressure and flow resistances back to values prevailing before section of the nerves This was essentially so as far as arterial pressure and flow resistances were concerned but the bradycardia elicited by cardiac nerve stimulation was however much more pronounced than would be expected if only normalization" occurred This finding suggested that fibres not normally tonically active were excited by artificial electrical stimulation Such fibres appear to converge particularly on efferent vagal *neurons since* atropinization of the animal markedly attenuated the bradycardial response to cardiac nerve stimulation

A more detailed analysis of the peripheral vascular adjustments to cardiac nerve stimulation indicated that the renal vascular bed was more preferentially engaged in reflexes elicited from cardiac nerves than from carotid sinus baroreceptor reflex me-

chanisms This agrees with the finding that interruption of vagal afferent activity produced most marked effects on the renal circulation The intestinal vessels on the other hand were engaged to approximately the same extent in the two types of reflexes while skeletal muscle vessels were more strongly influenced from the baroreceptors It is thus evident that the two inhibitory reflex mechanisms under study show differencies with regard to the distribution of the efferent outflow to the various cardiovascular effectors

The cardiac receptors have been postulated to exert a particularly strong influence on the capacitance vessels (Neil 1962) The present experiments have shown that the veins are indeed engaged in reflexes elicited from cardiac afferents The magnitude of the response was however similar to that obtained with arterial baroreceptor stimulation It was furthermore impossible to elicit a reflex venous response without a concomitant resistance vessel response irrespective of the characteristics of the stimulation applied to the cardiac nerves Thus the experiments do not support the hypothesis of a particularly strong or selective engagement of the veins in reflexes elicited from heart receptors

The functional role of cardiac receptor reflexes in circulatory control must remain a matter of speculation since the adequate stimulation for cardiac receptors is not known with certainty There are indications however that the extent of stretch of atrial muscle fibers determines the firing rate of type B atrial receptors (Paintal 1953) and the degree of filling of the circulation might thus be signalled to the central nervous system It therefore seems reasonable to assume that such a volume sensing system would be activated by *e g* blood loss and then in conjunction with the arterial baroreceptors initiate adequate compensatory measures The unloading of the heart receptors following hemorrhage should then according to the results of the present study mainly initiate a renal vessel constriction and a tachycardia It is possible that this tachycardia and consequent reduction in stroke volume and pulse pressure then diminish arterial baroreceptor activity even in face of an unchanged mean blood pressure to induce fluid reabsorption from the skeletal muscles (Öberg 1964) By these means blood volume can be replenished without gross interference with arterial pressure and blood flow through vital organs

The engagement of the renal vessels when cardiac receptor activity is reduced also seems to be an appropriate volume regulating mechanism Little is known however if and to what extent the glomerular filtration rate is altered by the renal vasoconstriction It is possible however that the changes in renal hemodynamics lead to alterations in salt excretion by activation of the renin angiotensin system and therefore in the long term influence extracellular fluid volume To settle these questions detailed studies on local renal hemodynamics are necessary

The circ

prevailing needs Such a cardio-cardiac reflex mechanism would then constitute some sort of "protective" reflex, preventing overloading of the heart The marked bradycardia obtained in the present study with stimulation of normally non-tonic cardiac afferent fibres might very well constitute such a protective reflex

This research has been sponsored in part by the AIR FORCE OFFICE OF SCIENTIFIC RESEARCH through the European Office of Aerospace Research, OAR United States Air Force under Contract F61052-68-C-044, by US Public Health Service Research Grant HE 05675-07 and by the Swedish Medical Research Council (K68-14X-644 03 and 04)

References

AMANN, A. and H SCHAEFER, Über sensible Impulse im Herznerven *Pflugers Arch ges Physiol* 1943 *246* 757—789

AVIADO, D M, T H LI, W KALOW, C F SCHMIDT, G L TURNBULL, G W PESKIN, M E HESS and A J WEISS, Respiratory and circulatory reflexes from the perfused heart and the pulmonary circulation of dog *Amer J Physiol* 1951 *165* 261—277

BEZOLD, VON, A and L HIRT, Über die physiologischen Wirkungen des essigsauren Veratrins *Untersuch Physiol Lab Wurtzburg* 1867 *1*

DALY I DE BURGH and E B VERNEY, Cardiovascular reflexes *J Physiol* (Lond) 1926 *61* 268—274

FOLKOW, B O LUNDGREN and I WALLENTIN, Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 *57* 270—283

GAUER, O H and J P HENRY Circulatory basis of fluid volume control *Physiol Rev* 1963 *43* 423—481

GUAZZI M A LIBRETTI and A ZANCHETTI Tonic reflex regulation of the cat's blood pressure through vagal afferents from the cardiopulmonary region *Circulat Res* 1962 *11* 7—16

HEYMANS C and E NEIL *Reflexogenic areas of the cardiovascular system* J & A Churchill Ltd London, 1958

JARISCH A and Y ZOTTERMAN Depressor reflexes from the heart *Acta physiol scand* 1948 *16* 31—51

KOLATAT T G ASCANIO R J TALLARIDA and M J OPPENHEIMER, Action potentials in the sensory vagus at the time of coronary infarction *Amer J Physiol* 1967 *213* 71—78

MELLANDER S Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat *Acta physiol scand* 1960 *50* Suppl 176

NEIL E, Neural factors responsible for cardiovascular regulation *Circulat Res* 1962 *11* 137—140

ÖBERG B Effects of cardiovascular reflexes on net capillary fluid transfer *Acta physiol scand* 1964 *62* Suppl *229*

ÖBERG B and P THORÉN To be publ *1970*

PAINTAL A S A study of right and left atrial receptors *J Physiol* (Lond) 1953 *120* 596—610

PAINTAL A S The study of ventricular pressure receptors and their role in the Bezold reflex *Quart J exp Physiol* 1955 *40* 89—111

PAINTAL, A S *Vagal afferent fibres Ergebnisse der Physiologie* Springer Verlag 1963 *52* 74—156

PEARCE J W and J P HENRY Changes in cardiac afferent nerve fibre discharges induced by hemorrhage and adrenalin *Amer J Physiol* 1955 *183* 650

PILLSBURY, *H R C M GUAZZI and D FREIS Vagal afferent depressor nerves in the rabbit Amer J Physiol* 1969 *217* 768—770

ROSS, J JR C J FRAHM and E BRAUNWALD Influence of intracardiac baroreceptors on venous return, systemic vascular volume and peripheral resistance *J clin Invest* 1961 *40* 563—572

RUSHMER, R F B L FINLAYSON and A A NASH Shrinkage of the heart in anesthetized [illegible] 1954 *2* 22—27

[illegible] RIEBEN Reflex effects of left ventricular distension [illegible]

[illegible] *fferent cardiac and pulmonary fibres* Abstract, XIX [illegible]

Acta physiol. scand. 1970. 80. 395—403

From the Department of Physiology, University of Göteborg, Sweden

The Role of Vagal Cardiac Nerves and Arterial Baroreceptors in the Circulatory Adjustments to Hemorrhage in the Cat

By

B Öberg and S White[1]

Received 23 March 1970

Abstract

Öberg, B and S White *The role of vagal cardiac nerves and arterial baroreceptors in the circulatory adjustments to hemorrhage in the cat* Acta physiol scand 1970 *80* 395—403

The relative importance of arterial baroreceptors and receptors with afferent fibres in the cardiac nerves, respectively, in producing compensatory circulatory adjustments in moderate hemorrhage was analyzed in chloralose-anesthetized cats — The effects of standardized hemorrhage on blood pressure, heart rate and renal and skeletal muscle blood flows were observed before and [illegible]

vaso-vagal' syncope in man This response was found to be mediated through a vago-vagal reflex arch and probably constitutes a protective mechanism causing a break on the heart in situations of extremely poor diastolic filling

In a previous study (Öberg and White 1970) the effects of section and stimulation of vagal cardiac afferent fibres on the resting circulation of the chloralose-anesthetized cat were studied in some detail The results suggested that the tonic rhythmic activity known to occur in cardiac afferents (*e g* Whitteridge 1948, Paintal 1953, 1955) probably exerts a generalized inhibitory influence on central vasomotor neurones, but the effects were rather moderate when compared with those of arterial baroreceptors The studies also indicated that the tonic restraint was mainly directed to the control of heart rate and vasomotor tone in the kidney It was also shown that low frequency stimulation of the vagal cardiac afferent fibres resulted in profound

[1] Overseas Research Fellow of the Life Insurance Medical Research Fund of Australia and New Zealand *Present address* Hallstrom Institute of Cardiology Royal Prince Alfred Hospi Missenden Rd Camperdown, 2050, N S W , AUSTRALIA

bradycardia, which was probably initiated in sensory nerves not tonically active unde resting conditions

The present experiments were undertaken to investigate in a more physiologic way the possible role of heart receptors in circulatory control Since the receptor have been ascribed a volume regulation function (*e g* Gauer and Henry 1963) it wa *a priori* assumed that alterations of blood volume by means of hemorrhage and re transfusion of the shed blood would provide an adequate and natural way to un load and activate the receptors Attempts were also made to activate receptor signalling through the suspected non tonic afferents by means of vary rapid hemor rhage thus simulating the situation of "vaso vagal" syncope in man

Methods

Experiments were performed on 20 cats (mean weight 2 8 kg, range 2 4—3 6 kg) anesthetized with chloralose 30—50 mg/kg b w — The carotid arteries and vagal nerves were dissected free on each side in the neck and separated from each other for a long distance The aortic nerves were identified and cut at the junction with the superior laryngeal nerve In some ani mals the sinus nerves were dissected free bilaterally and placed on ligatures for later section The thorax was opened with bilateral intercostal incisions and positive pressure breathing ad ministered while the cardiac nerves on both sides were freed and placed on ligatures as de scribed in a previous paper (Öberg and White 1969) The abdomen was then opened and the intestine removed The left renal vein was dissected free for cannulation [illegible] led on a Crass [illegible] pressure pulse

Hemorrhage was usually carried out through a catheter placed in the superior mesenteric artery or sometimes through a c [illegible] gular vein The bleeding was either [illegible] minutes or rapid where ap [illegible] Each experiment started with [illegible] cardiac vagal nerves intact Identical hemorrhage were then again repeated 3—5 times after interruption of the afferent pathways from one or both receptor sites by cutting the sinus or/ and cardiac nerves respectively In some experiments cardiac afferent activity was interrupted by cooling the vagi in the neck This was simply done by packing frozen saline around the nerves insulated from surrounding structures by plasticine troughs These animals were usually atropinized prior to cooling (0 3—0 5 mg i v) to eliminate the effects of interrupted efferent vagal influence on the heart

Results

1 *Circulatory effects of moderate hemorrhage*

The effects of moderate hemorrhage on blood pressure heart rate and flow re sistances in skeletal muscle and kidney were studied in 11 animals with aortic nerves cut In one group consisting of 5 animals (group A) standardized hemorrhages were carried out before and after cutting the cardiac nerves or cooling the cervical vagi but with the carotid sinus baroreceptors intact — In the remaining 6 animals (group B), hemorrhage was performed before and after section of the sinus nerves In the latter situation the cardiac nerves probably constitute the only remaining important

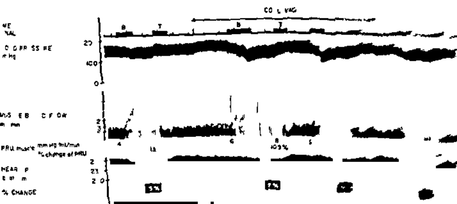


Fig 1 Cat 2 8 kg Chloralose 30 mg/kg body weight

afferent source of sensory information from the circu
centre To test this reflex adjustments to hemorrhage
animals after avulsion of the cardiac nerves

The circulatory response of group A animals to m
ml of blood withdrawn during 60—120 sec are shown
responses to hemorrhage before and after eliminati
show that the circulatory adjustments are only affec
arterial baroreceptor function is normal The differen
in *e g* skeletal muscle and renal vascular beds in th
the responses obtained with vagal cooling or card
There was however differences in heart rate both
tive point of view in the two cases Thus with card
increase in heart rate developed very early during b
withdrawal was complete and often before any s gr

TABLE I Circulatory responses to standardized hemorrh
diac receptor afferents Sinus nerves intact thr
are expressed as per cent change from pre bleed
tute means of means of 3—6 bleedings in 5 an

	Bleeding (Car sinus barorec + heart rec ntact)	Cooling (or avulsi card ac ner
Heart rate	10 ± 1 1	2 ± 0
Blood pressure	—35 6 5	4 ± 0 8
Muscle PRU	43 ± 15 2	8 ± 1 8
Renal PRU	39 10 1	11 ± 1 7

TABLE II Circulatory responses to standardized hemorrhages before and after interruption of arterial baroreceptor and cardiac nerve afferents Aortic nerves cut The responses are expressed as per cent change from pre bleed control and constitute means of means of 3—5 bleedings in 6 animals

	Hemorrhage with sinus nerves and cardiac nerves intact	*Sinus nerves cut*	*Hemorrhage with cardiac nerves intact sinus nerves cut*	*Avulsion of cardiac nerves*	*Hemorrhage with cardiac and sinus nerves cut*
Heart rate	6±2 1	2±1 4	7±1 8	3±1 4	0
Blood pressure	−29±4 5	35±6 8	−38±5 3	10±2 4	−52±2 0
Muscle PRU	75±20 2	51±14 6	30±18	8±1 6	8±2 6
Renal PRU	42±14 7	5±1 7	65±6 4	40±1 6	9 4±3 5

pressure occured With hemorrhage after interruption of the vagal afferents, the tachycardia developed slowly and did not reach maximum values until the arterial pressure was significantly reduced This difference in time course with regard to the development of tachycardia in the two situations is illustrated in Fig 1 This finding suggests that the heart receptors are of importance for inducing heart rate changes during hemorrhage of such a small magnitude that arterial blood pressure is not significantly altered (Guapta *et al* 1966)

It was further evident that the per cent increase in heart rate was consistently higher when the animal was bled with intact cardiac afferents The less pronounced per cent increase in heart rate with hemorrhage after section of cardiac afferent fibres is partly explained by the relative tachycardia present already before the start of the hemorrhage and caused by elimination of tonic cardio inhibitory impulses in vagal afferents (Table I) (Öberg and White 1970) However with intact cardiac afferents the heart rate sometimes increased more also in *absolute* terms (Fig 1) than after section or cooling of the afferents This was particularly evident in two of the five animals in this series where the inhibitory restraint from the cardiac afferents seemed to be rather weak to judge from the fact that heart rate did not increase noticeably when the cardiac nerves were cut These animals thus showed decidedly less intense increases in heart rate with hemorrhage after section of cardiac afferents This finding seems to indicate that not only *inhibitory* but also *excitatory* impulses travel in cardiac afferent fibres The presence of an excitatory mechanism, emanating from the heart and directed to the regulation of heart rate has indeed recently been described (Ledsome and Linden 1967)

In the presence of normally functioning arterial baroreceptor reflexes the contribution of the vagal cardiac nerves to the compensatory cardiovascular adjustments to blood loss therefore seems rather small possibly except for their effects on heart rate However when the sinus nerves were cut leaving cardiac receptors as probably the only remaining cardiovascular receptor site of importance their potency in compensating for blood loss was found to be quite marked This is illustrated in Table II, where data from 6 animals in group B are collected When compared with the responses obtained when hemorrhage was carried out with sinus nerves as well as

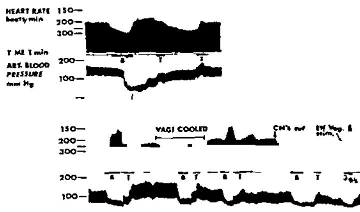


Fig 2 Cat 3 2 kg Chloralose 40 mg/kg body weight.

Effects of bleeding (B) and transfusion (T) on heart rate and blood pressure in the closed chest animal (upper record)and when thorax was opened and the cardiac nerves dissected free (lower record)

The upper record shows that with an initial slow hemorrhage blood pressure is well maintained *and heart rate increases* When the *rate* of blood withdrawal is *suddenly increased* With all of vagi gi (3 f the

card ac nerves

vagal cardiac nerves intact the magnitude of heart rate and renal responses was just *as marked or even more pronounced when only cardiac nerves remained intact The* responses of the skeletal muscle vascular bed was however less marked in the latter situation — After avulsion of the cardiac nerves compensatory responses to hemorrhage were very small or even absent — These findings show that cardiac receptors can indeed efficiently combat the harmful effects on the circulation of moderate blood loss They will in contrast to the baroreceptors affect mainly the heart and the renal vasculature and to a less extent the skeletal muscle vascular bed

2 *Circulatory effects of rapid hemorrhage*

When bleeding was carried out very rapidly (20—30 ml in 15—20 sec) a quite different *type of response pattern was often though not always obtained After an* initial tachycardia in the beginning of the bleeding period a marked bradycardia suddenly appeared reducing the heart rate far below the resting pre bleeding" values (Fig 2) — The bradycardia sometimes vanished spontaneously, but it could also be reversed to a marked tachycardia with retransfusion of part of the shed blood This latter response thus showed a close resemblance to the so-called Bainbridge effect. — The bradycardia of rapid bleeding could be eliminated by cooling the cervical vagi proving its reflex nature The response was also abolished with

indicating that vagal fibres constitute the efferent pathway in the reflex. Finally, th response was also eliminated by cutting the cardiac vagal nerves (Fig 2) This was due to interruption of the *afferent* and not the efferent reflex pathway, since e ferent stimulation of the cervical vagi was in this situation still capable of eliciting marked reduction in heart rate (Fig 2) Thus, it is likely that the reflex bradycard of rapid blood loss has its origin *in the heart and is essentially dependent on a vag vagal reflex loop*

Discussion

The circulatory adjustments following hemorrhage, are the result of widespread ac tivation of autonomic effector mechanisms as well as the local and mechanical effec of blood volume loss (Chien 1967, Chalmers, Korner and White 1967 a and b) Th arterial baroreceptors are considered the main source of the reflex activity (Heyman and Neil 1958) but there is also evidence that arterial chemoreceptors play a rol (Colderidge, Kenney and Neil 1949 Kenney and Neil 1951, Landgren and Ne 1951) but probably only when the blood loss is severe (Chalmers *et al* 1967 a, Or Kiwull and Wiemer 1967) It has also been suggested that the scattered mechano receptors of the heart and pulmonary regions also take part (Langrehr and Krame 1960, Gauer and Henry 1963, Öberg 1963 Gupta Henry, Sinclair and von Baum garten 1966) Little is known however with regard to the relative contribution o the receptors discussed above nor of the role of central mechanisms in relation to the more peripherally placed receptor groups

The present study helps to resolve some of these questions, at least for blood losse of approximately 10 % of the blood volume over one to two min. The results show that both arterial baroreceptors and receptors with their afferents in the vagal cardiac nerves of the cat contribute and work in a complimentary fashion to produce the compensatory adjustments to hemorrhage The fact that vasomotor effects were es sentially absent when the afferent input from carotid sinus, aortic and cardiac vagal nerves was eliminated indicates that these cardiovascular receptor mechanisms are the chief determinants of the reflex response to hemorrhage The small residual ef fects might be ascribed to ischaemia of medullary (Sagawa *et al* 1961) or spinal (Alexander 1945) vasomotor neuron pools following acute reduction in cardiac out put and arterial pressure

Even if tachycardia and constrictions of renal and skeletal muscle vessels could be induced from either set of afferent fibres during hemorrhage, the patterns of response differed in some respect indicating that the two receptor groups pursued their "com pensatory" function utilizing somewhat different efferent vasomotor pathways. This was evident when each afferent source was operating in the absence of the other Thus, when the circulatory responses to hemorrhage was entirely directed through the arterial baroreceptors a rather uniform activation of the vasoconstrictor fibres to the kidney and the skeletal muscles and of the accelerance fibres to the heart seemed to take place When on the other hand receptors with afferents in the cardiac nerves

were left alone to combat a moderate loss, the compensation occurred mainly by a strong activation of the accelerance fibres and the constrictor fibres to the kidneys The superimposition of the two reflex response patterns account well for the observed overall cardiovascular adjustment to hemorrhage

The present data support earlier presented more indirect evidence that receptors with their afferents in the vagi seem to be of special importance for producing reflex shifts in heart rate in hemorrhage (Öberg 1963) The results also resemble those obtained with carotid occlusion and interruption and stimulation of cardiac vagal nerves (Öberg and White 1970) in that receptors, served by the vagal cardiac nerves appear preferentially concerned with the control of renal vasomotor tone and heart rate, and to a lesser extent, flow resistance in skeletal muscle

The present studies do not define the receptors served by the cardiac vagi, or their site Electrophysiological studies of action potentials in atrial receptor afferents indicate a marked reduction in firing rate during moderate hemorrhage (Langrehr and Kramer 1960, Gupta Henry, Sinclair and von Baumgarten 1966), suggesting that these receptors may take part in the reflexes initiated through the vagal cardiac nerves The extent or importance of pulmonary afferents in these nerves is not known (Paintal 1963)

The findings extend recent studies related to hemorrhage in the unanesthetized rabbit (Chalmers *et al* 1967 b) In these experiments the widespread vasoconstriction following loss of a quarter of the blood volume was greatly modified in animals with previous section of the carotid sinus and aortic nerves However, vasoconstriction still occurred in the kidney and since the vagi were intact in these animals such an effect might be expected to follow reflexes initiated from the region of the heart The reason for the absence of an increase in heart rate following blood loss in the same preparations is not clear but it may be related to the already very high cardiac rate in this species following carotid sinus and aortic nerve section Reflex regulation of the renal bed from afferents situated in the central circulation has been suspected for some time (Arndt Reineck and Gauer 1963 Gauer and Henry 1963) The present studies indicate that this is certainly so but whether this is a specific reflex from a particular receptor group within the heart and/or lungs with afferents in the cardiac vagi remains to be investigated

The pronounced reflex slowing of the heart obtained with rapid blood withdrawal and evidently elicited from heart receptors resemble in many respects the bradycardia produced by electrical stimulation of supposedly non tonic vagal cardiac afferents (Öberg and White 1970) and the vasovagal reaction in man (*e g* Barcroft and Swan 1953) It has indeed been proposed that this latter response is initiated from heart receptors stimulated when the ventricular muscle is vigorously contracting on an empty chamber (Henry 1955 Sharpy Schaefe 1956) The present findings [illegible] support this hypothesis It seems likely that with rapid loss of circulating fluid a [illegible] tion arises where an extremely poor diastolic filling of the heart is combined [illegible] strong sympathetic excitation of the myocardium and that receptors [illegible] active become stimulated during such extreme conditions (*e g* Pearce [illegible]

1955). A strong reflex excitation of vagal efferents to the heart will then constitute an effective break on the pump and permit a better diastolic filling and possibly an improvement of the pumping efficiency of the heart. Moreover, with the increased diastolic filling the squeezing and rubbing of the ventricular walls, assumed to occur when inadequately filled ventricles beat violently and manifesting itself in subendocardial hemorrhages (Henry and Gauer 1954) is avoided. This cardio-cardiac inhibitory reflex can therefore be looked upon primarily as a protective reflex by which cardiac dynamics and the efficiency of the pump can be adjusted to match the extent of diastolic filling, particularly in situations with a critically reduced venous return.

This research has been sponsored in part by the AIR FORCE OFFICE OF SCIENTIFIC RESEARCH through the European Office of Aerospace Research, OAR, United States Air Force under Contract F61052 69-C-044, by U.S. Public Health Service Research Grant HE-05675-07 and by the Swedish Medical Research Council (K69-14X-644-03 and 04).

References

ALEXANDER, R. S., The effects of blood flow and anoxia on spinal cardiovascular centers. *Amer. J. Physiol.* 1945. *143*: [illegible]
ARNDT, J. O., H. REINECKE and O. H. GAUER, Ausscheidungsfunktion und Hämodynamik der Nieren bei Dehnung des linken Vorhofes am narkotisierten Hund. *Pflügers Arch. ges. Physiol.* 1963. [illegible]: 1–15.
BARCROFT, H. and H. J. C. SWAN, *Sympathetic control of human blood vessels.* Edward Arnold & Co., London 1953. 120–155.
[illegible], J. P., P. I. CORNER and S. W. WHITE, The effects of haemorrhage on the [illegible] and rabbit. *J. Physiol. (Lond.)* [illegible]. *189*: [illegible]
[illegible], J. P., P. I. CORNER and S. W. WHITE, Effects of haemorrhage on the [illegible] blood flow in the rabbit. *J. Physiol. (Lond.)* [illegible]. *193*: [illegible]
[illegible], Role of the sympathetic nervous system in haemorrhage. *Physiol. Rev.* [illegible]
[illegible], J. C. G., R. A. KENNEDY and E. NEIL, Evidence of the contribution of the chemoreceptor [illegible] McDowall reflex. *J. Physiol. (Lond.)* [illegible]
GAUER, O. H. and J. P. HENRY, Circulatory basis of fluid volume control. *Physiol. Rev.* [illegible]
GAUER, O. H. and J. P. HENRY, [illegible]
[illegible]
HENRY, J. P., [illegible]
[illegible], C. and E. NEIL, [illegible] of the cardiovascular system. J. & A. Churchill, London [illegible]
KENNEDY, R. A. and E. NEIL, [illegible] haemorrhage. *J. Physiol. (Lond.)* [illegible]
LANDGREN, S. and E. NEIL, Chemoreceptor [illegible] following haemorrhage. [illegible]
[illegible], D. and K. KRAMER, [illegible]
[illegible], F. R. and R. J. LINDEN, [illegible]
ÖBERG, B., [illegible]

ÖBERG B and S W WHITE Circulatory effects of interruption and stimulation of cardiac vagal afferents *Acta physiol scand* 1970 *80* 383—394

OTT, N P KIWULL and W WIEMER Die Wirkung der Vagus und Depressorausschaltung auf dem Blutdruck bei normalen sowie erniederigtem Ausgangsdruck *Pflugers Arch ges Physiol* 1967 *294* R11

PAINTAL A S A study of right and left atrial receptors *J Physiol* (Lond) 1953 *120* 596—610

PAINTAL A S, The study of ventricular pressure receptors and their role in the Bezold reflex *Quart J exp Physiol* 1955 *40* 89—111

PAINTAL, A S, Vagal afferent fibres *Ergebn Physiol* Springer Verlag 1963 *52* 74—156

PEARCE J W and J P HENRY, Changes in cardiac afferent nerve fibre discharges induced by [illegible]

Acta physiol. scand 1970 80 404—411

From the Department of Pharmacology, Karolinska Institutet Stockholm Sweden

Fate of ^{3}H-Noradrenaline in Canine Subcutaneous Adipose Tissue[1]

By

Bertil B Fredholm and Sune Rosell

Received 23 March 1970

Abstract

Fredholm B B and S Rosell *Fate of ^{3}H-noradrenaline in canine subcutaneous adipose tissue* Acta physiol scand 1970 *80* 404—411

Canine subcutaneous adipose tissue was isolated and perfused with defibrinated blood at a constant rate Infused ^{3}H noradrenaline was taken up by the tissue and released spontaneously into blood Following a washout period of 40 min or more, plasma samples were withdrawn Radioactive compounds in venous plasma were separated by chromatography on alumina and Dowex About 30 per cent of the venous radioactivity was identified as unchanged noradrenaline about 7 per cent as normetanephrine and 14 per cent as deaminated catechols deaminated O methylated metabolites accounted for about 45 per cent This indicates the presence in adipose tissue of both monoamineoxidase and catechol O methyl transferase Upon stimulation of sympathetic nerves to adipose tissue peripheral resistance increased the ^{3}H in adipose tissue of both monoamine oxidase and catechol O methyl transferase Upon [illegible] noradrenaline [illegible] uced [illegible] e of [illegible] ased [illegible] he

associated with vascular reactions in adipose tissue

There is good evidence that the sympathetic nervous system plays an important role in the regulation of fatty acid mobilization from adipose tissue (see reviews by Havel 1965 1968)

The metabolism of the adrenergic transmitter noradrenaline in adipose tissue appears to be incompletely known In a comparative study Stock and Westermann (1963) found appreciable amounts of monoamine oxidase (MAO) in white adipose tissue but no detectable catechol O methyl transferase (COMT) activity Recently however Traiger and Calvert (1969) demonstrated COMT activity in rat epididymal adipose tissue using a more sensitive method

With these observations as a background the present experiments were undertaken to study the metabolism of ^{3}H noradrenaline during basal conditions and following

[1] Part of this work has been presented at the XXI Scandinavian Pharmacological Meeting Acta Pharmacol (Kbh) 1967 25 suppl 4 20

nerve stimulation in canine subcutaneous adipose tissue *in situ* (Rosell 1966) It is known that infused noradrenaline is taken up by the adrenergic nerve terminal system and released following nerve stimulation (Hertting *et al* 1961, Rosell, Kopin and Axelrod 1963). Therefore, ^{3}H-noradrenaline was used as an indicator of the fate of the endogenous transmitter

Methods

The experiments were conducted on 8 female mongrel dogs The subcutaneous adipose tissue situated between the pubis, the abdominal midline and the right inguinal ligament was freed

[illegible] Blood was drawn from the femoral

[illegible] The tissue was perfused at a constant

[illegible] 962) The venous outflow passed via a

[illegible]

pressure and perfusion pressure were measured by Statham pressure transducers and recorded, together with the blood flow rate, on a Grass Polygraph

After the start of the perfusion, dl 7-^{3}H noradrenaline (^{3}H NA) (5C/mM, New England [illegible]

the reservoir

FFA were determinated in arterial and venous samples according to Dole (1956) as modified by Trout *et al* (1960) Glycerol was determined enzymatically according to Laurell and Tibbling (1966) The release or uptake of FFA and glycerol was calculated from the arteriovenous difference multiplied by the plasma flow per 100 g of adipose tissue

For the determination of total venous radioactivity, 0 2 ml aliquots of plasma were transferred to counting vials and the radioactivity was determined as described below For the [illegible]

[illegible] were eluted in sequence from the column by 1 M HCl

In the other experiments the procedure was modified to include absorption of catechols [illegible]

[illegible]

separation procedure The substances were assayed using their native fluorescence except for NA which was determined according to Haggendal (1963 The recovery of NA following adsorption on alumina averaged 82 per cent (76—91) and approximately 40 per cent of the DOMA was recovered in the alumina eluate The recovery of NA on the Dowex column averaged 76 per cent and 82 per cent of the NM was recovered The appearance of radioactivity in the various fractions was followed after running plasma samples containing ^{3}H-NA through the separation procedure By these means corrections for losses and contami[illegible] could be made

The metabolite pattern in venous blood was further analyzed in 2 expts using

) (Änggård Sjoqvist and Sjostrom 1970 Ang as deproteinized as above The extracts were to a short Dowex 50—X4 column The combined neutral and acid metabolites The former were he latter at pH 2 The amines were eluted from the column by 1 M HCl in me derivatives (Änggard Sedvall with diazomethane These as on Sephadex LH 20 columns method NA NM VMA DC the presence of *DHPG* During and immediately following nerve stimulation for 30 min about 40 per cent of the radioactivity in the perchloric acid extract was accounted for by amines NA being more abundant than NM about 20 per cent as acids with more VMA than DOMA about 15 per cent as neutrals *i e* MHPG About 30 per cent of the radioactivity remained in the aqueous phase after extractions with the organic solvents at pH 7 and pH 2 The results obtained with this method were thus similar to those reported below and offer were made by the use of external standardization

Estimation of radioactivity

Whole or part of the acid eluate or combined effluent and washing from the Dowex columns were evaporated to dryness *in vacuo* at 35° C The dry residues were thereafter dissolved in 15 ml of 0 4 % 2 4 diphenyloxazole and 0 005 % 1 4 di(2,5 phenyloxazole) benzene (*POPOP*) in equal parts of toluene and glycolmonoethylether containing 5 % methanol The radioactivity was measured in a Packard liquid scintillation spectrometer Corrections for counting efficiency were made by the use of external standardization

Results

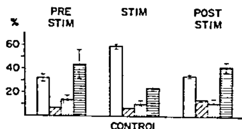
Release of radioactivity during basal conditions

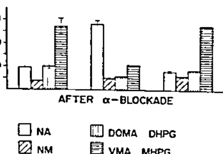
The pattern of radioactivity in venous blood 50—70 min after an infusion of dl ^{3}H NA *is shown in the upper left hand part of Fig 1 It can be seen that* 32± 3 per cent of total radioactivity appeared as unchanged NA 7±0 5 per cent as NM d that 14+3 per cent occurred as deaminated catechols (DOMA DHPG) This ows that both O methylation and oxidative deamination does take place in canine adipose tissue

Release of radioactivity following sympathetic nerve stimulation

Fig 2 illustrates a typical result Upon nerve stimulation with 10 cps the perfusion pressure increased indicating an arteriolar vasoconstriction During the stimulation there was a marked rise in the outflow of ^{3}H NA into venous plasma At the same time the outflow of deaminated O methylated NA metabolites decreased The outflow of deaminated catechols (DOMA DHPG) did not change significantly As a consequence the total outflow of radioactivity was hardly changed during the stimulation After cessation of the stimulation the outflow of ^{3}H NA rose further before returning to the prestimulatory level The outflow of the ^{3}H NA metabolites also increased following cessation of the stimulation This appears especially significant for the metabolites which had been metabolized by COMT

In the lower tracing of Fig 2 the release of glycerol from the adipose tissue is shown There was a decrease in the release rate of glycerol during the stimulation However, following cessation of the stimulation the release rose A latency in glycerol release has been described previously Fredholm and Rosell 1968





Relative proportions of ^{3}H noradrenaline and its metabolites 10 min before, during and 10 min after nerve stimulation with or without pretreatment with 100 ug dihydroergotamine The values are the means ±S E M of the relative proportions obtained in individual experiments NA and NM values in the control situation are based on 6 expts the values deaminated metabolite proportions are derived from 4 expts After α blockade prestim NA NM, 5 expts, deaminated metabolites 3 expts, stim and poststim NA NM 4 expts, deaminated metabolites 2 expts

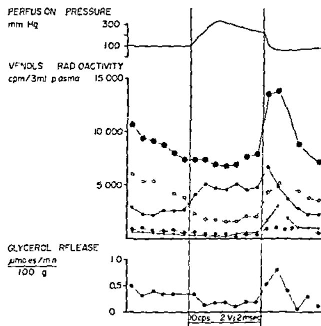
In the other 5 expts where the nerve was stimulated with frequencies between 3 and 6 cps, qualitatively the same responses were obtained

The net effect of the changes in the pattern of radioactivity during nerve stimulation is illustrated in the upper middle part of Fig 1 NA constituted 59±2 NM 7±0 7, DOMA DHPG 10±3 and VMA MHPG, 24±2 per cent of total radioactivity respectively This means a significant increase ($p<0 01$) in NA and a significant decrease in VMA MHPG ($p<0 01$) The pattern of radioactivity following cessation of the nerve stimulation is illustrated in the upper, right hand part of Fig 1 It is apparent that the post stimulatory pattern is very similar to the prestimulatory one The only definite change is in the relative proportion of NM which has increased from 6 to 14 per cent ($p<0 05$)

Effects of dihydroergotamine

Fig 3 illustrates 1 expt out of 5 where dihydroergotamine was given Prior to dihydroergotamine nerve stimulation produced essentially the same changes as described above *i e* a rise in noradrenaline outflow and a diminished outflow of deaminated O methylated metabolites After cessation of the stimulation the release of radioactivity increased A pronounced vasoconstriction was also produced as seen from the perfusion pressure recording The i a administration of 100 ug of dihydroergotamine had no obvious effect *per se* on the outflow of radioactivity

When the stimulation was repeated the response pattern was changed The total outflow of tritium radioactivity increased substantially during the stimulation period and returned rapidly to the prestimulatory release rate following cessation stimulation The increase in total tritium was almost completely accounted for

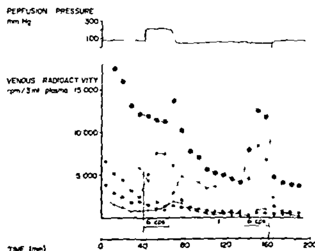


The effect of nerve stimulation (10 cps, 12 V, 2 msec for 2 min) on perfusion pressure outflow of ^{3}H NA and metabolites and on glycerol outflow in canine subcutaneous adipose tissue perfused with defibrinated blood at a constant rate (4 8 ml/min/100 g)

250 µC ^{3}H noradrenaline was administered by constant infusion during 25 min Samples were withdrawn 50 min after cessation of the noradrenaline administration Samples were withdrawn at 3 min intervals ●——● Total radioactivity, ○- - ○ deaminated O methylated ^{3}H metabolites, ⊗——⊗ ^{3}H noradrenaline, ×——× ^{3}H normetanephrine, ● - ● deaminated ^{3}H metabolites

increased outflow of NA There was also a small increase in the outflow of NM This was of minor quantitative importance however The outflow of deaminated metabolites (DOMA DHPG VMA, MHPG) showed no clearcut change After hydroergotamine nerve stimulation caused a vasodilatation

The other 4 expts where the same dose of dihydroergotamine (100 µg) and stimulation frequencies between 3 and 6 cps were used gave similar results The pattern of radioactivity following dihydroergotamine before, during and after nerve



The effect of nerve stimulation (6 cps 12 V 2 msec for 24 min) before and after i a administration of 100 µg dihydroergotamine (↑) on perfusion pressure release of ^{3}H noradrenaline and its metbolites in canine subcutaneous adipose tissue perfused with defibrinated blood at a constant rate (6 1 ml/min/100 g)

250 µC ^{3}H noradrenaline was administered by constant infusion for 25 min Samples were withdrawn at 8 min intervals starting 60 min after the end of the ^{3}H noradrenaline infusion ●——● Total plasma radioactivity ○ ○ deaminated O-methylated ^{3}H metabolites, ○——○ ^{3}H noradrenaline, ×——× ^{3}H normetanephrine ●- - - -● deaminated ^{3}H metabolites

stimulation is given in the lower part of Fig 1 It can be seen that before the stimulation the pattern is essentially similar to that seen during the control period with the exception that the percentage of noradrenaline was lower than during the control period 18 ± 2 and 31 ± 3 respectively The difference is statistically significant ($p < 0\,01$) but may only reflect changes with the time as will be discussed

During nerve stimulation the pattern of radioactivity was similar in the two situations (Fig 1) It must be emphasized however that the background to the induced changes was different Thus before α receptor blockade the relative decrease in VMA MHPG was due to an absolute decrease whereas after dihydroergotamine the relative decrease was due to a more pronounced increase in other radioactive products (see also Fig 3) Following cessation of the nerve stimulation the release of radioactivity returned to the prestimulatory pattern Analogously to the control situation the relative amount of NM showed a slight elevation (from 7 to 12 per cent) which was statistically insignificant however

In all 5 expts the outflow of radioactivity upon nerve stimulation was increased (30—135 per cent) following pretreatment with dihydroergotamine ($p < 0\,01$) In the 2 expts where the outflow of products of lipolysis was estimated FFA release was potentiated by 175 and 60 % respectively and glycerol release by 240 % and 190 %

Discussion

The present data indicate that the degradation of the adrenergic transmitter substance in canine subcutaneous adipose tissue follows the same enzymatic pathways as in other tissues *e g* skeletal muscle Thus both O methylated and deaminated metabolites of ^3H noradrenaline were found in the venous blood Experiments on rat epididymal fat pads and isolated fat cells have revealed the presence of MAO (Stock and Westermann 1963) and COMT (Traiger and Calvert 1969)

In spite of the fact that a washout period of 40—60 min was used equilibration was apparently not complete as is seen from the fact that in all experiments the basal outflow rate of radioactivity decreased with time The fact that endogenous and ^3H NA stores were not in equilibrium sets a limitation on the quantitative conclusions concerning the metabolism of transmitter substance in adipose tissue Dihydroergotamine was always given late in the experiments The fact that a smaller proportion of noradrenaline was recovered in the venous effluent following dihydroergotamine than during control conditions (Fig 1) might indicate a change in the distribution of the ^3H NA during the experiment The fact that the injection of dihydroergotamine *per se* apparently caused only slight changes in the metabolic pattern (Fig 3) inforces this interpretation

The effects of nerve stimulation on the outflow of ^3H NA and its metabolites were similar to these reported for skeletal muscle (Rosell Kopin and Axelrod 1963) Thus the ^3H NA content in the venous blood rose upon nerve stimulation wh the deaminated metabolites decreased This decrea e lends further support

opinion that sympathetic nerve stimulation hinders the transport of a variety of solutes from tissue to blood, presumably due to a diminished capillary surface area following pre-capillary sphincter closure as discussed elsewhere (Rosell 1969, Linde and Rosell 1970, Fredholm, Öberg and Rosell 1970) Presumably, the transport of NA is also restricted The high rate of ^{3}H-NA release from adrenergic nerves during stimulation may however, obscure this effect in the present experiments

Using the histochemical fluorescence technique of Falck and Hillarp, Wirsen (1964) was unable to demonstrate adrenergic nerve endings in direct contact with the white fat cells Similarly recent electronmicroscopic work has failed to reveal synapses on these cells (Napolitano 1965) It is therefore possible that the adrenergic transmitter substance has to pass from the nerve terminals around the blood vessels to the fat cells in order to exert its lipolytic action Provided that a part of the released noradrenaline reaches the fat cells via the blood stream the constriction of arterioles as well as pre-capillary sphincters during nerve stimulation could severe the transport of noradrenaline to the fat cells This could partly explain the finding that the release of FFA and glycerol seen upon nerve stimulation is delayed (Rosell 1966 Fredholm and Rosell 1968 Fig 2 present report) In addition there is strong evidence that FFA and glycerol are trapped within the tissue during the stimulation period (see Rosell 1969)

Traiger and Calvert (1969) found that part of the adipose tissue COMT activity is localized at the adipocyte cellmembrane That COMT is not closely associated with the nerve ending has also been proposed by Axelrod (1966) The observed decreased outflow of O methylated metabolites might thus conceivable reflect a smaller transmitter concentration at the enzyme site possibly the fat cell due to impaired transport of released noradrenaline Following adrenergic α receptor blockade nerve stimulation produced a vasodilatation rather a vasoconstriction and probably there was less impairment of the transport of noradrenaline to the fat cell and to the extraneuronal enzymes degrading it in particular COMT

The importance of the vascular effects of nerve stimulation for potentiation of transmitter overflow after α receptor blockade was pointed out by Rosell Kopin and Axelrod (1963 Rapid diffusion of transmitter substance away from its site of release would tend to increase transmitter overflow due to diminished local inactivation. The total outflow of transmitter substance would thus be greater following α receptor blockade and more of it could reach the receptor sites on the fat cells The finding that adrenergic α receptor blockade potentiates the lipolytic response to nerve stimulation Fredholm and Rosell 1968 may be explained along these lines

This investigation was supported by grants from Swedish Medical Research Council (B70-14X-731-05A) C. S Johnsons fond at Svenska Läkaresällskapet and from Karolinska Institutet which are gratefully acknowledged

Our thanks are due to Dr Erik Änggård for providing the facilities for and to Miss Berit Holmberg for carrying out the liquid gel chromatographic separation of noradrenaline metabolites The skilful technical assistance of Miss Mona Engqvist is also gratefully acknowledged

References

ÄNGGÅRD, E, G SEDVALL and B SJÖQVIST, Chromatography of biogenic amine metabolites on lipophilic sephadex II The catecholamines and their O methylated derivatives *J Chromatogr* In press

ÄNGGÅRD, E, B SJOQVIST and R SJOSTROM, Chromatography of biogenic amine metabolites on lipophilic sephadex I The phenolic acids and alcohols *J Chromatogr* 1970 *50* 251—259

ANTON, A H and D F SAYRE, A study of the factors affecting the aluminium oxide-trihydroxyindole procedure for the analysis of catecholamines *J Pharmacol. exp Therap* 1962 *138* 360—375

AXELROD, J, Methylation reaction in the formation and metabolism of catecholamines and other biogenic amines *Pharm Rev* 1966 *16* 95—113

CA[illegible]

FR[illegible]

Therap 1968 *159* 1—7

FREDHOLM, B B, B ÖBERG and S ROSELL, The effect of some vasoactive compounds on the series coupled sections in canine subcutaneous adipose tissue *Acta physiol scand 79* 564—574

HÄGGENDAL, J, An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues *Acta physiol scand* 1963 *59* 242—254

HAVEL, R J, Autonomic nervous system and adipose tissue In *Handbook of Physiology*, ed by A E Renold and G F Cahill, Jr, sect V pp 575—582, Williams and Wilkins Co, Baltimore, 1965

HAVEL, R J, The autonomic nervous system and intermediary carbohydrate and fat metabolism *Anaesthesiol* 1968 *29* 702—713

HERTTING G, J AXELROD, I KOPIN and L G WHITBY, Lack of uptake of catecholamines after chronic denervation of sympathetic nerves *Nature* (Lond) 1961 *189* 66

LAURELL, S and G TIBBLING, An enzymatic fluorimetric micromethod for the determination of glycerol *Clin chim acta* 1966 *13* 317—322

LINDE, B and S ROSELL To be published

NAPOLITANO, L M, Observations on the fine structure of adipose cells *Ann N Y Acad Sci* 1965 *131* 34—42

RENKIN, E M and S ROSELL The influence of sympathetic adrenergic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in skeletal muscle *Acta physiol scand* 1962 *54* 223—240

ROSELL S Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation *Acta physiol scand* 1966 *67* 343—351

ROSELL S Nervous and pharmacological regulation of vascular reactions in adipose tissue In *Drugs affecting lipid metabolism* (Eds W L Holmes L A Carlson and R Paoletti) *Exp Med Biol* 1969 *4* 24—34 Plenum Press New York.

ROSELL S I J KOPIN and J AXELROD Fate of H^3 noradrenaline in skeletal muscle before and following sympathetic stimulation *Amer J Physiol* 1963 *205* 317—321

STOCK K and E WESTERMANN Concentration of norepinephrine serotonin and histamine and of amine metabolizing enzymes in mammalian adipose tissue *J Lipid Res* 1963 *4* 297—304

TRAIGER G J and D N CALVERT O methylation of ^3H norepinephrine by epididymal adipose tissue *Biochem. Pharmacol* 1969 *18* 109—117

TR[illegible] A study

W[illegible] icroscopy

Acta physiol. scand. 1970. 80. 412—419

From the Second Department of Pathology, University of Helsinki, Finland

In Vitro Assay of the Stimulatory Effect of Hypoxic Serum on Cellular Proliferation in the Erythron

By

E. KIVILAAKSO

Received 2 April 1970

Abstract

KIVILAAKSO, E. *In vitro assay of the stimulatory effect of hypoxic serum on cellular proliferation in the erythron.* Acta physiol. scand. 1970. 80. 412—419.

The effect of experimental hypoxia on mitotic homeostasis in the erythron was investigated by means of an *in vitro* technique. The proliferative responses were measured in terms of ^3H-thymidine incorporation in normal rat bone marrow cells *in vitro*. Test serum was collected from rats kept in hypoxic conditions. The hypoxic serum caused a significant (32 %) stimulation of total incorporation of ^3H-thymidine in the target cells as compared to normal serum. Autoradiographic analysis revealed that this effect was due to an increase in the number of bone marrow cells labelled. The stimulation proved to be tissue-specific: the number of labelled erythrocyte precursors was increased by 45 %, while the effect on the granulocyte population was negligible. Gel filtration of the hypoxic and normal sera on Sephadex G 200 revealed that the effect was exerted by a stimulatory factor present in the hypoxic serum, there being no substantial difference between the two sera in their contents of the inhibitor chalone. Elution parameters of the stimulator on Sephadex G 200 suggested a molecular weight in the region of 60 000—70 000. As judged from the molecular weights, this stimulator and erythropoietin may be identical, but the exact relation of the two factors cannot be deduced with certainty on the basis of the data available. The results are discussed.

It is by now firmly established that the balance between erythrocyte supply and demand is regulated by humoral feedback mechanisms which control the influx from a self-maintaining stem cell population and hence the mitotic activity and rate of maturation of the precursor cells in the bone marrow. The central component in this control system is obviously erythropoietin, which seems to act by inducing differentiation of the stem cells, which then augment the erythron (Alpen and Cranmore 1959, Erslev 1959). Whether it also acts on the precursor cells beyond the stem cell compartment is not yet unambiguously established.

Recent experimental data suggest that in erythropoiesis stem cell induction is not the only mechanism of control, but that the rate of cellular proliferation in the erythron is regulated independently of erythropoietin. From mature erythrocytes it has been possible to extract a tissue-specific antimitotic factor, termed the erythrocytic chalone, which has an inhibitory influence on mitotic proliferation in the

erythron (Kivilaakso and Rytomaa 1970b) In physiological conditions this inhibitor is probably liberated continuously from the circulating erythrocytes into the surrounding plasma and thus the rate of erythrocyte production can be adjusted to meet incidental changes in the number of circulating cells

In the present study an attempt has been made to shed further light on the mechanism by which maintenance of mitotic homeostasis in the erythron is achieved the main question being whether a mitotic stimulator analogous to the granulocytic antichalone (Rytomaa and Kiviniemi 1967 1968a, b) is involved in the regulation of red cell production in conditions associated with enhanced erythropoises In a short term *in vitro* assay system the erythropoietin dependent influx from the stem cell compartment is negligible and thus it has been possible to obtain a direct measure of the proliferative responses of the pre existing precursor cells of the marrow

Material and methods

Hypoxic serum

Rats (adult Sprague Dawley males weighing about 300 g) were kept for 18 hrs in low oxygen tension under normal barometric pressure by maintaining through the cages (large glass jars) a constant flow of a gas mixture composed of 7—8 % oxygen and 92—93 % nitrogen After this treatment the sera were collected by cardiac puncture and stored at −18° C until used

Gel filtration technique

(chalone free) normal serum served as basic culture med um in the subsequent assay cultures

Bioassay technique

The effects of the test sera or filtration fractions on bone marrow cell proliferation were measured in terms of ^{3}H thymidine incorporation in the cells *in vitro* Details of the short term assay system adopted have been described elsewhere (Rytomaa and Kiviniemi 1967 Rytomaa 1969 Kivilaakso and Rytomaa 1970a)

Target bone marrow cells were obtained from the femora of untreated rats and an equal number of cells was added to the test and control cultures The culture medium in experiments with whole sera consisted of 20 % test or control serum respectively and 80 % Hanks BSS ^{3}H thymidine (sp act 3 0 Ci/mmole The Radiochemical Centre Amersham England) was added to the cultures at zero time to give a concentration of 1 μCi/ml The cells were incubated stationary at + 37° C as a monolayer" on a cover glass hanging from the top of a tissue culture tube After incubation for a predetermined time a set of four essentially identical cover glass cultures was harvested from the test and control groups respectively and prepared for analy s as described before (Kivilaakso and Rytomaa 19 0a) T[illegible] corporated in whole cultures were measured in a Pack by putting the cover glass on the bottom of a vial 100 % of the radioactivity measured is present in the PCA hydrolysis (Rytomaa 1969 Kivilaakso unpublished results)

After measurement of the total radioactivities some of the cover glass preparations were washed in a series of solutions of dioxan and water to remove the scintillation liqu autoradiograms were then prepared by the Kodak AR 10 stripping film tec proc es ed autoradiograms were then stained in dilute Giemsa or Mayer [illegible] was decolorized at acid pH

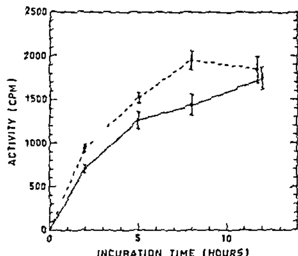


Fig 1 Cumulative incorporation of ^{3}H thymidine in normal rat bone marrow cells *in vitro* Mean ±SE of 4 experimental (o - - - o) and of 4 control (o——o) cultures are presented at each incubation time The activities

was +41 1

Results

Total incorporation of ^{3}H-thymidine into the cells

The effect of the hypoxic serum on total incorporation of ^{3}H-thymidine in rat bone marrow cells was studied in eight independent experiments (256 individual cultures) The time course curve of ^{3}H-thymidine accumulation in the cells in 1 expt is shown in Fig 1 The relative effects at different incubation times, expressed as percentage deviation from the control are presented in Table I

The results demonstrate that the hypoxic serum has a distinct stimulatory effect on ^{3}H-thymidine incorporation in the cells In an analysis of variance carried out on the raw data in each individual experiment, both main variables type of serum and incubation time proved to be statistically significant the combined signifi

TABLE I Effect of hypoxic serum on the incorporation of ^{3}H thymidine in normal rat bone marrow cells *in vitro*

Experiment	Relative effect (deviation from the control) Incubation time 2h	5h	8h	12h	Mean
I	4 1	35 9	39 5	35 0	+28 6 %
II	+35 1	29 3	6 1	32 0	+25 6 %
III	9 5	14 8	9 5	11 0	+ 6 5 %
IV	−31 5	20 6	36 2	+ 5 9	+23 6 %
V	−13 7	11 0	33 7	+30 6	+15 4 %
VI	13 9	23 4	33 8	+13 3	+21 1 %
VII	+42 0	24 6	9 5	+14 5	+22 7 %
VIII	+55 0	16 2	29 5	+20 0	+30 2 %
				+21 7 2 7 % (mean ± SE n = 8)	

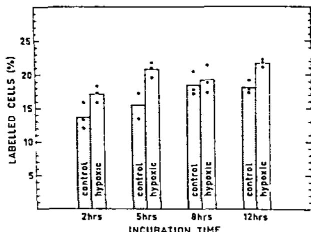


Fig 2 Labelling of normal rat bone marrow cells *in vitro* in the presence of hypoxic and control sera Each column represents the mean value of 3 individual cultures shown as black dots

cances of different experiments were P<0 001 for both sets of variables (Van Valen 1964) In contrast to this, interaction between the two main variables was insignificant in each experiment The absence of biological interaction between the main variables can also be deduced from Table I, for the length of the incubation time did not have any systematic influence on the magnitude of the stimulation (analysis of variance, F<1) This in turn suggests that the hypoxic serum does not influence the relative rate of ^{3}H thymidine incorporation into the cells *i e* the rate of DNA synthesis

As the length of the incubation time did not systematically affect the magnitude of the stimulation it was possible to pool the relative effects observed within each experiment at different incubation times (Table I) The average stimulation of all experiments was 21 7 ± 2 7 % (mean ± SE, n = 8), which differs significantly from zero at the level P<0 001 (Student's t test)

Autoradiographic analysis

In order to obtain deeper insight into the mechanism by which hypoxic serum exerts its stimulatory effect after one experiment (expt VI in Table I) the cover glasses were subjected to autoradiographic analysis The first step was to determine the overall proportion of bone marrow cells labelled by examining 500 nucleated bone marrow cells from each sample The labelling indices for the individual samples are shown in Fig 2

It is seen that the overall labelling index tended to increase with the incubation time being about 20 % higher (P<0 001, analysis of variance) in the test cultures than in the control cultures This suggests that the hypoxic serum had triggered the entry of a cohort of resting precursor cells into the generative cell cycle

Subsequently the 5 and 8 hr preparations were subjected to a more detaile analysis The questions to be answered were (i) did the hypoxic serum increase

TABLE II An autoradiographic analysis of the effects of hypoxic and control sera on normal rat bone marrow cells *in vitro* The control and experimental groups are composed of three 5 and 8 hr preparations respectively (thus n = 6)

Group	n	Grain count	Labelled precursor cells (per 100 bone marrow cells) erythrocytes	granulocytes	erythrocytes + granulocytes
control	6	27 5±0 9	7 4±0 4	9 6±1 0	17 0±1 0
hypoxic	6	28 3±1 8	10 7±0 6	8 9±0 5	19 6±0 8
Group difference		+2 9 %	+44 6 %	−7 3 %	+15 3 %
Significance (Student s t test)		insign	P<0 001	insign	P<0 05

amount of ^{3}H thymidine incorporation *per cell* and (ii) was the effect tissue specific to the erythrocyte precursor cells

The first problem was approached by means of a grain count analysis by making a random count of the number of grains in the nuclei of ten labelled normoblasts in each sample (Table II) It is seen that there was no significant difference between the two groups which indicates that the stimulatory effect of the hypoxic serum did not result from an increase in the amount of ^{3}H thymidine *per cell*

It is thus apparent that the way in which the hypoxic serum stimulated ^{3}H thymidine incorporation was by increasing the number of labelled precursor cell (Fig 2) The tissue specificity of this action was studied by determining the proportion of labelled erythrocyte and granulocyte precursor cells respectively in the test and control cultures Since in a short term incubation the differential cell count is essentially the same in all cultures the labelled cells are expressed per 100 nucleated bone marrows cells rather than in terms of specific labelling indices (Table II)

The results demonstrate that the stimulatory effect of the hypoxic serum was exerted on the erythrocyte precursor cells only The number of labelled erythrocyte precursors increased by 44 6 % (P<0 001 Student s t test) while the effect on granulocyte precursors was insignificant

Partial purification of the stimulatory factor on gel filtration

Nextly samples of the hypoxic and fresh normal sera were subjected to gel filtration on Sephadex G 200 in order to separate the active substances from the bulk of the other components of the sera In each experiment 10 equal sized fractions were collected and tested immediately as regards their effects on total incorporation of ^{3}H thymidine into normal rat bone marrow cells *in vitro* using incubation times of 4 and 9 hours The pooled results of three replicate filtrations of hypoxic and normal sera respectively are presented in Fig 3

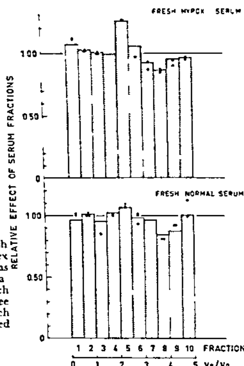


Fig 3 Fractionation of hypoxic and fresh normal serum by gel filtration on Sephadex G 200 The effectiveness of the fractions was measured in terms of ^{3}H thymidine incorporation into rat bone marrow cells *in vitro* Each column represents the mean value of three repeated tests shown as black dots In each test the baseline level (1 00) was determined from several untreated cultures

It is seen that fraction 5 in the hypoxic serum was highly stimulatory It thus appears that the stimulatory effect of the hypoxic serum resulted from the appearance of a stimulatory agent in the serum in hypoxic conditions In gel filtration on Sephadex G 200 this agent had a relative elution volume (V_e/V_o) of about 2 0 ($K_{av}\sim$ 0 4) which is suggestive of a molecular weight in the region of 60 000—70 000 As seen from Fig 3 the stimulatory effect of the corresponding fraction of normal serum does not differ much from the baseline level but judging from the similar tendency towards stimulation small amounts of the stimulator may be present even in normal serum

Fig 3 also reveals that both sera contained inhibitory components which in the present assay system were eluted in fractions 7—9 It is known from previous experience that this elution zone contains the two mitotic inhibitors of bone marrow cells the erythrocytic and granulocytic chalones (Kivilaakso and Rytomaa 1970b Rytomaa and Kiviniemi 1968a) The relative elution volumes of these factors on Sephadex G 200 seem to be in the region 3 0—3 5 ($K_{av}\sim$1 0) which is indicative of a relatively small molecular material This observation is consistent with the earlier estimates of the molecular weight of the chalones in question which have fallen within the range of 2000—4000 (Rytoma and Kiviniemi 1968a Kivilaakso and Rytomaa 1970b) No substantial difference seems to exist between the chal contents of the two sera as judged from Fig 3

Discussion

The results obtained in the present study demonstrate that in hypoxic conditions and possibly also in other conditions in which the demand for erythrocytes is acute a tissue specific mitotic stimulator appears in rat serum In contrast to this the concentration of chalone in the serum seems to remain unchanged Thus the situation is somewhat different from that found in granulocytopoiesis where a stimulator, the granulocytic antichalone, seems to replace rather than counteract the effect of chalone in the serum (Rytomaa and Kiviniemi 1968a)

The mode of action of the present mitotic stimulator may be analogous to that of the granulocytic antichalone and antagonistic to that of the erythrocytic chalone Its primary action seems to be to promote the entry of the resting erythrocyte precursor cells into the DNA synthesis phase and presumably also to start the actual generative cell cycle In contrast to this it, like the chalones, does not affect the speed at which the cells go through the cycle, as judged from the absence of time serum interaction in the cumulative incorporation of ^{3}H thymidine and from the grain count analysis *of the autoradiograms From the point of view of population kinetics the factor thus* increases erythrocyte production by enhancing proliferation efficiency within the erythron one stem cell would now produce a greater number of mature erythrocytes The possibility that the same factor also possesses additional functions not manifested in the present assay system is of course not excluded

As judged from gel filtration the stimulatory factor seems to be a relatively large molecule with a molecular weight of about 60 000—70 000 It is thus easily separated by means of gel filtration from the erythrocytic and granulocytic chalones and obviously also from the granulocytic antichalone whose molecular weight has been estimated to be about 30 000—35 000 (K_{av}~0 6 on Sephadex G 200) (Rytomaa and Kiviniemi 1968a) In contrast to this its molecular size seems to be of the same magnitude as that of erythropoietin whose molecular weight has been estimated to be approximately 62 000 by means of gel filtration on Sephadex G 200 Olesen and Fogh 1968)

The exact relation of the present mitotic stimulator to erythropoietin cannot be deduced with certainty from the available data Several authors have reported that erythropoietin has a stimulatory action on the mitotic activity or rate of DNA synthesis in the erythron (e g Powsner and Berman 1967 Dukes 1968 Necheles et al 1968) but others have failed to demonstrate this (Alpen et al 1959 Erslev 1964 Krantz and Goldwasser 1965 One possible explanation for the controversy could be that the relatively impure erythropoietin preparations may contain one or more active components This possibility is supported by the observation that unpurified anaemic or hypoxic serum/plasma seems to have a definite stimulatory action on the rate of proliferation of erythrocyte precursors as judged from the data presented here and in previous studies (e g Kuna et al 1959 Bernardelli 1962 Matoth and Kaufman 1962)

From the evidence presented here and in previous studies (Kivilaakso and Rytomaa 1970a, b), it is plain that as regards the control of cellular proliferation, the

erythron is basically similar to other tissues (see Bullough and Rytomaa 1965 Bullough 1965) In normal steady state conditions the proliferation of the erythrocyte precursors seems to be controlled mainly by the chalone as a negative feedback and in terms of tissue mass (cell numbers) In response to an acute demand for erythrocytes however, a tissue specific mitotic stimulator seems to become the dominant factor in rat serum It is possible that as proposed in a previous paper (Kivilaakso and Rytomaa 1970b) minor fluctuations in the demand for erythrocytes are primarily met by changes in the mitotic activity of the erythron and that induction of additional erythrocyte precursors from stem cells only becomes necessary in more stressfull situations

References

Bullough W S Mitotic and functional homeostasis A speculative review *Cancer Res* 1965 *25* 1683—1727

Bullough W S and T Rytomaa Mitotic homeostasis *Nature* (Lond) 1965 *205* 573—578

function in perfused isolated hind legs of rats *Amer J Physiol* 1959 *196* 769—774

Matoth Y and L Kaufman Mitotic activity in vitro of erythroblasts previously exposed to erythropoietin *Blood* 1962 *20* 165—172

Necheles T R Sheehan and H Meyer Studies on the control of haemoglobin synthesis Nucleic acid synthesis and normoblast proliferation in the presence of etrythropoietin *Ann N Y Acad Sci* 1968 *149* 449—455

Olesen H and J Fogh Apparent molecular weight of erythropoietin determined by gel filtration *Scand J Haemat* 1968 *5* 211—214

Powsner E R and L Berman Effect of erythropoietin on DNA synthesis by erythroblasts in vitro *Blood* 1967 *30* 189—197

Rytomaa T Granulocytic chalone and antichalone *In Vitro* 1969 *4* 47—58

Rytomaa T and K Kiviniemi Regulation system in blood cell production in *Control of Cellular Growth in Adult Organisms* p 106—138 Ed by H Teir and T Rytomaa Academic Press London 1967

Rytomaa T and K Kiviniemi Control of granulocyte production I Chalone and anti ... *Ce ... ue Kinet* 1968a *1* 329—340

R ... locyte production II Mode of action of ... *1* 341—350

... significance tests *Nature* (Lond) 196... 201 64

Acta physiol. scand 1970. 80. 420—425

From the Institute of Physiology University of Oslo Norway

Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish

I. The Existence of a Double Inhibitory Feedback

By

J K S Jansen, A Njå and L Walløe

Received 3 April 1970

Abstract

Jansen J K S A Njå and L Walløe *Inhibitory control of the abdominal stretch receptors of the crayfish I The existence of a double inhibitory feedback* Acta physiol scand 1970 *80* 420—425

By stretch activation of the slowly adapting abdominal stretch receptor of the crayfish we have found reflex activation of two efferent spikes of different amplitudes in the dorsal nerves of the same and neighbouring segments The large as well as the small efferent spike inhibit the discharge of the receptor It is suggested that these two units represent the thick and thin accessory fiber described by Alexandrowicz as making synaptic contacts with the receptor cell

To determine the signal transfer properties of chains of nerve cells simple systems with well defined input and output signals should be profitable at the present stage of knowledge With this in mind we have examined certain proprioceptive reflexes of the crayfish abdomen The preparation offers several advantages Firstly the abdominal stretch receptors has been extensively studied since their original description by Alexandrowicz (1951) and the pioneering studies of their functional properties particularly by Kuffler (1954) and Eyzaguirre and Kuffler (1955 a b) A second useful feature is the small number of nerve cells involved The entire slow extensor muscle is innervated by six axons and their spikes can to a large extent be identified individually in records of the activity of the dorsal nerve (Fields 1966 Fields *et al* 1967

Kuffler and Eyzaguirre (1955) demonstrated the inhibitory nature of the thick accessory nerve fibre which makes synaptic contacts with the cell body and dendrites of the stretch receptors Eckert (1961 a b) found that this neurone was reflexly activated by the fast as well as the slowly adapting receptor of the same and neighbouring segments Later it was found by Fields and coworkers (Fields and Kennedy 1965, Fields 1966 Fields *et al* 1967) that one of the motoneurones of the slow extensor muscle was also activated by a stretch receptor input

The present paper reports certain qualitative aspects of these reflexes largely supporting the observations of previous investigators In addition a second inhibitory reflex, probably mediated by the thin accessory fibre of Alexandrowicz (1967) is described The distribution of these reflexes and their input-output relationships will be presented in subsequent papers The nomenclature of Alexandrowicz (1967) will be followed throughout

Methods

The experiments were performed on the isolated abdomen of fresh water crayfish (*Astacus fluviatilis*) The preparations were placed in oxygenated saline of the following composition NaCl 12 g/l KCl 0 4 g/l, $CaCl_2$ 1 5 g/l, $MgCl_2$ 0 25 g/l and buffered with tris to a pH of 7 2 to 7 4 The temperature of the bath was thermostatically controlled and kept at 12° C

Results

Stretch activation of the slowly adapting receptor may evoke efferent activity in several units of the dorsal nerve of the same and neighbouring segments Most consistently we have found activation of a medium sized spike with a latency of some 30 to 40 msec to a high frequency input signal An example is presented in Fig 1 which consists of simultaneous records of the activity of the dorsal nerves of the second (above) and third (below) abdominal segments Stretch activation of the slowly adapting receptor of the third segments is apparent from the occurrence of large afferent spikes in the lower record This induced firing of one unit in the neighbouring nerve The reflexly activated unit kept on firing as long as the stretch was maintained and stopped immediately at the release of the stretch In addition to this activity in the neighbouring nerve there was usually an activation of a similar small efferent spike in the input nerve

The nature of the reflex activity of the neighbouring segment appears from the experiment of Fig 2 which also contains simultaneous records of dorsal nerve activity of two neighbouring segments A slight stretch of the receptor muscle caused a steady



Fig 1 Stretch activation of the slowly adapting receptor and the reflexly activated accessory fiber Top record Thick accessory fiber discharge recorded from dorsal nerve of second abdominal segment
Lower record Simultaneously recorded activity of ipsilateral dorsal nerve of third se The large spike is that of the slowly adapting receptor and its discharge indicates stre its receptor muscle *The small spike is reflex activity of the thick accessory fibre*

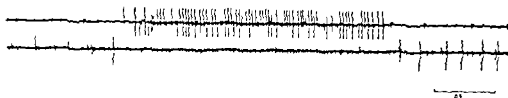


Fig. 2. The inhibitory nature of the reflex activity.

Lower record. Activity of the dorsal nerve of the third segment. Slight stretch of the receptor muscle gave a low rate background discharge of the slowly adapting receptor (large spike).

Top record. Simultaneously recorded activity of the ipsilateral second dorsal nerve. Stretch activation of the slowly adapting receptor (large spike). The receptor discharge of the top record activates the thick accessory fibre (lower record) which inhibits the receptor discharge of this segment.

afferent discharge (large spike) in the lower record. Stretch activation of the other receptor (large spikes, top record) activated reflexly a smaller spike which rapidly silenced the afferent activity in the lower record. This observation demonstrates the inhibitory nature of the reflexly activated spike of the neighbouring segments and is in complete agreement with Eckert's (1961 a) description of the accessory nerve reflex.

A second reflexly activated spike and further details of the effects appears from Fig. 3 which consists of four different segments of the records of dorsal nerve reflexes of neighbouring segments. Receptor activation in the top record (large spike Fig. 3A) excited two efferent spikes of different amplitudes in the lower record. Throughout Fig. 3A the larger and the smaller of the two spikes are marked with an arrow and an asterisk respectively. Sections B and C of Fig. 3 are from periods of maintained reflex activity and the two reflex spikes are readily apparent in the lower records of each section. Both these efferent spikes inhibited the discharge of the slowly adapting stretch receptor (largest spike). This appears from the intervals between impulses from the receptor. Without efferent activity the slowly adapting receptor fires with a high degree of regularity to steady stretch. In Fig. 3 the firing pattern of the receptor is less regular on account of the reflexly activated efferent spikes. The intervals in which such spikes occur are consistently longer than intervals without efferent spikes. Increased duration of receptor intervals are associated with the large as well as the small reflex spike. Particularly clear instances where one or the other of these spikes occur alone in an interval between receptor spikes are marked with the appropriate symbol in Fig. 3B and C. The prolongation of the receptor intervals are immediately apparent on comparison with preceeding or succeeding receptor intervals without efferent activity. Wherever the two efferent spikes occurred in the same interval their effects summated and the corresponding receptor interval was particularly long (Fig. 3C, middle of lower record). The reflex nature of the efferent activity appears from the silence after the release of the receptor (Fig. 3D lower trace).

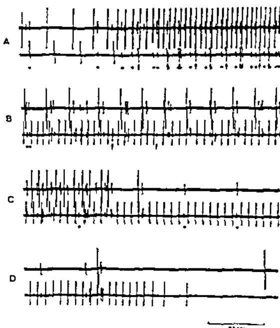


Fig 3 Reflex activation of both thick and thin accessory fibre Each pair of records show simultaneous activity of two neighbouring ipsilateral dorsal nerves. Large spike in all records are that of the slowly adapting receptors

A Increasing stretch of the receptor fibre of third segment (top record) activates large efferent spike (^) and small efferent spike (*) which inhibits the receptor discharge of the forth segment (lower record) B and C are different parts of the record of the same reflex which shows particularly clear examples of the inhibitory effect of the thick (^) and thin (*) accessory fibre D The efferent activity is silenced by the release of the receptor

An additional feature of the reflex ere is a high degree of coup This is most clearly seen in (*) in the dorsal nerve of a large efferent spike in the third segment (top record) This happened consistently when the

preparation In other experiments such coupling was much less pronounced or absent Since the recording situation has to be particularly favourable in order to observed the small reflex spikes we have unfortunately been unable to study the phenomenon more systematically We can therefore at present neither explain it in terms of possible mechanisms of coupling nor present reliable data on its frequency of occurrence

A further demonstration of the inhibitory effect of both the larger and smaller reflexly activated spikes is given in Fig 4A B These are plots of the increment of receptor intervals as a function of the time of occurrence of the inhibitory spike within the expected receptor interval For both large (Fig 4A) and small (Fig 4B) inhibitory spikes the delay of the succeeding receptor discharge is up to a certain limit, greater the later the inhibitory spike occurs within a receptor interval Inhibitory spikes occurring just before (less than some 5 msec) the expected time of a receptor discharge has no effect on the interval in which it occurs The shape of the curves of Fig 4 suggests that each of the reflex spikes after a delay (conduction time) has a inhibitory effect on the slowly adapting stretch receptor The inhibitory p

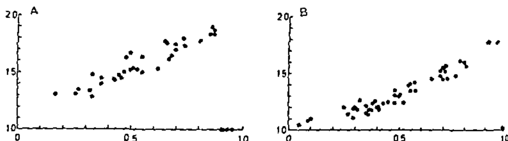


Fig 4 Plots of the increments of receptor intervals as a function of the time of occurrence of the inhibitory spike within the receptor interval for the large spikes (A) and for the small spikes (B)

Abscissa Normalized time of occurrence of an inhibitory spike within the expected inter spike interval of the stretch receptor Expected interval set equal to 1

Ordinate Ratio of duration of observed interval containing one accessory spike to duration of expected interval In the data used for A the range of expected intervals were 70—85 msec in B 80—100 msec

reaches an early maximum and decays progressively in the ensuing period The total duration of the inhibition after a single efferent spike is some 80 to 100 msec and in the interval range of Fig 4 both spikes optimally placed are able to increase the receptor interval by almost a factor of two A rather similar relationship has been obtained for the effect of the thick accessory fibre on the slowly adapting thoracic receptor by Perkel *et al* (1964)

The two inhibitory reflexes may be found in the stretch activated segment as well as in the neighbouring segments A third type of reflex spikes has been obtained only in the stretch activated segment itself In our experiments this third type of reflex has occurred less consistently than the two inhibitory reflexes mentioned above An example is given in Fig 5 Stretch activation of a slowly adapting receptor evokes different units in its own nerve One of the stretch activated units is marked with arrows in Fig 5 It has a medium sized nerve action potential which consistently is followed by a slower wave With our technique for en passage recording such spike wave complexes are commonly seen in the background activity of the nerve It is highly likely that this represents activity in excitatory motoneurones and that the wave part of the complex is due to the junction potentials of the muscle fibres Accordingly we find in agreement with Fields *et al* (1967) that one of the excitatory motoneurones may be activated from the slowly adapting receptor of the same segment

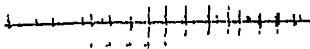


Fig 5 Stretch activated slowly adapting receptor (large spike) (A) followed by a slow wave Spikes of thick accessory fibre marked with asterisk Record from dorsal nerve of third segment

Discussion

There are two alternative and not necessarily mutually exclusive mechanisms by which the observed inhibitory effects could be mediated. The one is a direct inhibition of the receptor neurone by the accessory fibres and this has been demonstrated for the thick accessory fibre by intracellular records of IPSPs following its activation (Kuffler and Eyzaguirre 1955). The other is by inhibition of receptor muscle contraction assuming a background 'tonus' in this in our preparations. The short latency (Fig 4) and the restriction of inhibitory effects to the particular intervals in which accessory spikes occur are strong evidence against the latter hypothesis for both the large and the small accessory nerve effects. We conclude therefore that the slowly adapting stretch receptor is subjected to a double direct inhibitory control. A similar suggestion was made by Burgen and Kuffler (1957) for the thoracic stretch receptor.

The functional significance of the reflex activation of the large accessory fibre is at present enigmatic (Eckert 1961b, Fields *et al* 1967, Alexandrowicz 1967). The present observations does not contribute to its elucidation. On the contrary, the existence of a second reflex activated by the same input and with nearly the same peripheral effect has been established. One is indeed struck by the complexity of the proprioceptive machinery required to control the apparently simple abdominal movements of these animals. A further discussion of these reflexes requires a description of their distribution and quantitative relationships which will be given in subsequent papers.

References

ALEXANDROWICZ J S Receptor organs in the thoracic and abdominal muscles of crustacea *Biol Rev* 1967 *42* 288—306

ALEXANDROWICZ J S Muscle receptor organs in the abdomen of Homarus vulgaris and Palinurus vulgaris *Quart J micr Sci* 1951 *92* 163—199

BURGEN A S V and S W KUFFLER Two inhibitory fibres forming synapses with a single nerve cell in the lobster *Nature* (Lond) 1957 *180* 1490—1491

ECKERT R O Reflex relationships of the abdominal stretch receptors of the crayfish I Feedback inhibition of the receptors *J cell comp Physiol* 1961 a *57* 149—162

ECKERT R O Reflex relationships of the adbominal stretch receptors of the crayfish II Stretch receptor involvement during the swimming reflex *J cell comp Physiol* 1961b *57* 163—174

E

E

sile e neuro

FIELDS H L Proprioceptive control of posture in the crayfish abdomen *J exp Biol* 1966 *44* 455—468

FIELDS H L W H EVOY and D KENNEDY Reflex role played by efferent control of an invertebrate stretch receptor *J Neurophysiol* 1967 *30* 859—874

FIELDS H L and D KENNEDY Functional role of muscle receptor organs in the crayfish *Nature* (Lond 1965 *206* 1235—1237

KUFFLER S W Mechanisms of activation and motor control of stretch receptors of lobster and crayfish *J Neurophysiol* 1954 *17* 558—574

KUFFLER S W and C EYZAGUIRRE Synaptic inhibition in an isolated nerve cell *J gen Physiol* 1955 *39* 155—184

PERKEL D H J SCHULMAN T H BULLOCK G P MOORE and J P SEGUNDO P neurons effects of regularly spaced synaptic input *Science* 1964 *145* 61—63

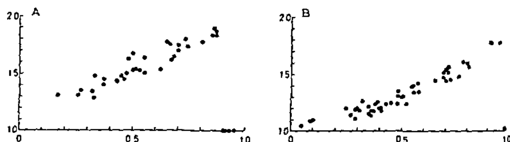


Fig 4 Plots of the increments of receptor intervals as a function of the time of occurrence of the inhibitory spike within the receptor interval, for the large spikes (A), and for the small spikes (B)

Abscissa Normalized time of occurrence of an inhibitory spike within the expected inter spike interval of the stretch receptor Expected interval set equal to 1

Ordinate Ratio of duration of observed interval containing one accessory spike to duration of expected interval In the data used for A the range of expected intervals were 60—85 msec in B 80—100 msec

reaches an early maximum and decays progressively in the ensuing period The total duration of the inhibition after a single efferent spike is some 80 to 100 msec and in the interval range of Fig 4 both spikes optimally placed, are able to increase the receptor interval by almost a factor of two A rather similar relationship has been obtained for the effect of the thick accessory fibre on the slowly adapting thoracic receptor by Perkel *et al* (1964)

The two inhibitory reflexes may be found in the stretch activated segment as well as in the neighbouring segments A third type of reflex spikes has been obtained only in the stretch activated segment itself In our experiments this third type of reflex has occurred less consistently than the two inhibitory reflexes mentioned above An example is given in Fig 5 Stretch activation of a slowly adapting receptor evokes different units in its own nerve One of the stretch activated units is marked with arrows in Fig 5 It has a medium sized nerve action potential which consistently is followed by a slower wave With our technique for en passage recording such spike-wave complexes are commonly seen in the background activity of the nerve It is highly likely that this represents activity in excitatory motoneurones and that the wave part of the complex is due to the junction potentials of the muscle fibres Accordingly we find in agreement with Fields *et al* (1967 that one of the excitatory motoneurones may be activated from the slowly adapting receptor of the same segment

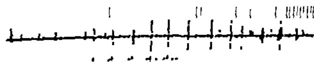


Fig 5 Stretch activated slowly adapting receptor (large spike) exciting efferent spike (A) followed by a slow wave Spikes of thick accessory fibre marked with asterisk Record from dorsal nerve of third segment

Two groups of rabbits were compared In one of the groups all the rabbits (9 animals) were about one year old In the other (5 animals) the ages varied between 3 and 5 years Differences between the 2 groups, as regards ejaculate volumes and libido could not be demonstrated However, the phosphatase activity of the seminal fluid from the younger rabbits was, on the average twice as great as that the seminal fluid from the older ones (Table I)

TABLE I The phosphatase activity (K. A) in seminal fluid from young and old rabbits respectively $\bar{x}$ = mean value of the phosphatase activity, s = standard deviation, n = number of determinations

	pH 4 9		pH 5 9	
	$\bar{x}$	s^2	$\bar{x}$	s^2
Young rabbits ($n = 14$)	173 4	3 749	156 3	3 283
Old rabbits ($n = 10$)	72 7 $t = 4\ 73$	1 033 $p < 0\ 001$	59 8 $t = 4\ 95$,	528 $p < 0\ 001$

The production of prostatic acid phosphatase (Gutman and Gutman 1939, ape and homo) as well as libido and ejaculate volumes (Cheng and Casida 1949, Parsons 1950), are considered to be dependent on androgens However, the ejaculate volumes and libido does not differ as between older and younger rabbits but the phosphatase activity does This suggests that there are different hormonal conditions for the production of seminal fluid and libido, on the one hand and phosphatase activity on the other Possibly the older rabbits have a lower androgen production If this be the case more androgen seems to be required for optimal phosphatase production and/or activity than for libido and ejaculate volume

The writer gratefully acknowledges the helpful advice of Professor Per Erik Lindahl
The investigation has been financially supported by Fonden för ograduerade forskares verksamhet Uppsala University

References

CHENG P and L E CASIDA Effect of testosteron propionate upon sexual libido and reproduction of semen and sperm in the rabbit *Endocrinology* 1949 *44* 38—48
GUTMAN E B E E SPROUL and A B GUTMAN Significance of increased phosphatase activity of bone at the site of osteoplastic metastases secondary to carcinoma of the prostate gland *Amer J Cancer* 1936 *28* 485—495
GUTMAN A B and E B GUTMAN Adult phosphatase levels in prepubertal rhesus prostate tissue after testosterone propionate *Proc Soc exp Biol* (N Y) 1939 *41* 277—281
KING E J and I D P WOOTTON Acid phosphatase estimation by determination of phenol with 4 aminoantipyrin A A P) Microanalysis in *Med Biochem* 1956 88—89 (Churchill Ltd London
KUTSCHER W and H WOLBERGS Prostataphosphatase *Hoppe-Seylers Z physiol Chem* 1935 *236* 237—240
PARSONS U Fructose in rabbit semen a study of normal fluctuations and changes evoked by testosterone and stilboestrol *J Endocr* 1950 *6* 412—42
SHULMAN S and J M FERBER Multiple forms of prostatic acid phosphatase *J Reprod Fertil* 1966 *11* 295—297
VERNON C A J GAULDIE J M HANSON J M HUMPHREYS P E SMITH, A J LAWRENCE and B F C BANKS Acid phosphatases *Nature* (Lond 1965 *208* 382—383

Acta physiol. scand. 1970. 80. 428—429

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Preferential Secretion of Newly Formed Noradrenaline in the Perfused Rabbit Heart

By

L. Stjärne and Å. Wennmalm

The concept of a rapidly turning over "secretory pool" of transmitter in sympathetic neurons and of the key role of newly formed transmitter in sustaining transmitter secretion during prolonged stimulation is mainly based on studies of the rate of formation and disappearance of noradrenaline (NA) in various animal tissues (Sedvall, Weise and Kopin 1968). Direct study of the fate of newly formed NA by means of perfusion techniques has been limited to the cat spleen and the results obtained are quite controversial. Thus, Kopin *et al.* (1968) working with the Krebs Ringer perfused cat spleen found that newly formed NA was secreted in distinct preference to preformed NA in response to nerve stimulation at a frequency of 30/sec and a duration of more than 2 min. On the other hand Blakeley *et al.* (1968) found no preferential secretion of newly formed NA in the cat spleen perfused with blood containing prostaglandin E_1 in response to repeated trains of 1000 stimuli delivered at 10/sec.

In the present experiments this issue was reexamined using the isolated sympathetically innervated rabbit heart, perfused according to the Langendorff technique with Tyrode solution containing 20 μg/ml of ascorbic acid and aerated with 6.5 % CO_2 in O_2. Contractile force and heart rate were recorded using conventional Grass equipment. ^{14}C labelled tyrosine (New England Nuclear Corp., uniformly labelled, S.A. 446 mC/mmole) and unlabelled tyrosine were infused into the heart in concentrations of 2.5 × 10 M (4 expts) or 2×10^{-5} M (9 expts). Five min after the start of the tyrosine infusion both right and left sympathetic nerve trunks were stimulated supramaxillary at a frequency of 5/sec (4 expts) or 10/sec (9 expts) for 4 or 6 min. At the end of the stimulation period the heart was rapidly homogenized in 0.4 M perchloric acid. Catechol compounds in the heart extract and in the perfusates were adsorbed on alumina, while non catechols, including tyrosine, were carefully removed by washing. Catechols in the alumina eluates were adsorbed on ion exchange columns (Amberlite CG 120, sodium form, 0.4 × 80 mm). After removing the acid including DOPA and catechol metabolites by washing with 0.25 M sodium acetate pH 4, NA and dopamine (DA) were separated by chromatography using 1 M HCl as eluant. NA was determined fluorimetrically and the radioactivity of 1 ml aliquots of the different fractions was counted in 10 ml of Instagel solution (Packard Co.) in a Packard Liquid Scintillation Spectrometer. Both NA levels and radioactivity were low, and only those chromatograms in which there was good agreement between the profiles of radioactivity and fluorimetrically determined NA were accepted.

The results will be presented in more detail elsewhere. The main features observed

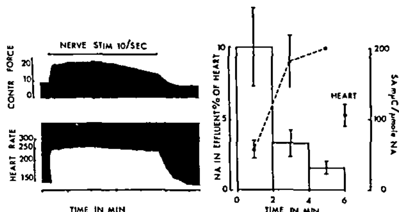


Fig 1 Perfused rabbit heart Left inotropic and chronotropic response to prolonged supra maximal stimulation at 10/sec of the sympathetic nerves to the heart. Right outflow of NA (columns show mean ± S E M during 3 consecutive 2 min periods of nerve stimulation, n = 7, n = 7, n = 2) and specific activity (dotted line, mean ± S E M n = 7 n = 9 n = 1) in the perfusate Specific activity of the heart at the end of the experiments (mean ± S E M, n = 8) marked at the 6 min point

were the following The heart frequency response to nerve stimulation showed no fading during the stimulation periods while the contractile force response usually tended to decrease progressively However, the NA level in the effluent from the heart fell even more rapidly, particularly at 10/sec Thus the NA outflow did not show good correlation to the mechanical responses observed At the time when the NA level in the effluent had fallen to very low levels, the total amount of NA in the effluent amounted to about 15 % of the NA content of the heart at the end of the experiment During nerve stimulation the perfusate contained both labelled and unlabelled NA while no NA could be detected in the perfusate during the resting periods

The specific activity of NA in the effluent increased during the period when infusion of labelled tyrosine was combined with nerve stimulation, to reach a level 1 8 times higher than that in the heart (P = 3 64**, n = 8) during the 2 minute period immediately preceding the termination of the experiment indicating a distinct preference for secretion of newly formed NA

In conclusion, the present experiments are compatible with the view that the sustained secretion of transmitter response to prolonged stimulation of sympathetic neurons is to a considerable extent dependent on formation and preferential secretion of new transmitter molecules

This work was supported by grants from Magnus Bergvalls Stiftelse and from Stiftelsen Lars Hiertas Minne which are hereby gratefully acknowledged

References

BLAKELY A G H G L BROWN D P DEARNALEY and V HARRISON The effect of nerve stimulation on the synthesis of ^{3}H tyrosine in the isolated blood perfused cat spleen *J Physiol* Lond) 1968 *200* 59—60P

KOPIN I J G R BREESE K R KRAUSS and V K WEISE Selective release of newly synthesized norepinephrine from the cat spleen during sympathetic nerve stimulation *J Phar col exp Ther* 1968 *161* 271—287

[illegible] K WEISE and I J KOPIN The rate of norepinephrine [illegible]

Acta physiol. scand. 1970. 80. 430—432
From the Department of Pharmacology, University of Uppsala, Sweden

Colchicine-Induced Delay of the Degeneration Release of Sympathetic Transmitter in the Conscious Rat

By
DAG LUNDBERG

During the first few days following section of postganglionic autonomous nerve fibers (axotomy) there is a spontaneous and transient stimulation of the effector organ This effect is thought to be due to leakage of stored transmitter from the degenerating nerve terminals (for refs, see Lundberg 1969) The processes induced by the axotomy and responsible for the degeneration transmitter release are only slightly known Whatever these processes are the nerve terminals have to be informed of the fatal loss of the cell body In view of the present knowledge about the importance of intra-axonal flow of different proteins and other nerve constituents it is reasonable to suspect that the latency between axotomy and the degeneration transmitter release is related to intra-axonal transport of some essential material The "information could be the change in the flow of transported matter abruptly induced by the axotomy After a certain time lag which depends on the length of the nerve and the rapidity of the transport the 'information reaches the level of the nerve endings where processes responsible for the storage of the transmitter then are affected Colchicine is thought to be bound to specific microtubular proteins (Borisy and Taylor 1967) and has been found to inhibit intra-axonal flow of different constituents e g amine storage granules in sympathetic nerves of the rat (Dahlstrom 1968 acetyl choline esterase in sciatic nerve of the rat (Kreutzberg 1969) and protein in the rabbit optic nerve and tract (Karlsson and Sjostrand 1969) It therefore seemed worthwhile to test colchicine on a degenerating sympathetic nerve effector system which has been described in detail earlier (Lundberg 1969)

Male Sprague Dawley rats weighing about 250 g were used The magnitude of the palpebral aperture was measured hourly between 10 and 25 30 hrs after excision of the superior cervical ganglion on one side (denervation and preganglionic section of the sympathetic nerve on the other (decentralisation) A special optic apparatus allowing measurements on conscious animals was used The spontaneous and transient postdenervation increase in palpebral aperture due to contraction of the sympathetically innervated periorbital smooth muscle (the degeneration contraction) was thus followed Commercial colchicine was dissolved in saline and injected s c.

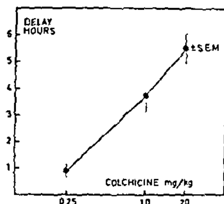


Fig 1 Effect of different doses of s c colchicine given at the time of denervation on the time of start of the degeneration contraction Delay is the difference between the T_{50a} of the experimental group and that of the control group T_{50a} is the time of 50 % development of the degeneration contraction The data are from table I

From Fig 1 and Table I it is obvious that colchicine given at the time of denervation delayed the onset of the degeneration contraction without appreciably changing its magnitude and duration However, when given at 12 hrs after denervation, which is about 3 hrs before the expected start of the contraction colchicine was ineffective Some rats in the 1 mg/kg group and all rats given 2 mg/kg had a certain loss of muscular tone, they were lethargic, and had diarrhoea The symtoms started at 20—25 hrs after denervation, but no rat died within 50 hrs after the injection which was the time of observation

TABLE I Effect of s c colchicine on the degeneration contraction T_{50a} is the time of 50 % development of the degeneration contraction T_{50d} is the corresponding level on the descending phase The values are means or means ± S E M if not otherwise stated Comparisons are between a treated group and the control group Student's t test was used for the analysis of significance Significance levels are *** = $p < 0.001$ ** = $p < 0.01$ and * = $p < 0.05$

Treatment design	n	Time of start (T_{50a}) hrs[1]	Delay (T_{50a} exp − T_{50a} cont) hrs	Duration T_{50d}—T_{50a} hrs	Peak aperture on denerv side mm
2.0 mg/kg at op	4	20.78 ± 0.60	5.54***	>8.64[2] n.t.	3.91 ± 0.42
1.0 mg/kg at op	9	18.99 ± 0.66	3.75**	10.23[2] n.t.	4.30 ± 0.11
0.25 mg/kg at op	5	16.17 ± 0.18	0.93	8.30 ± 0.86	4.55 ± 0.15
1.0 mg/kg at 12 hrs[1]	5	15.14 ± 0.86	−0.10	10.29 ± 1.69	4.49 ± 0.18
Controls (no drug given)	10	15.24 ± 0.26	—	8.73 ± 0.46	4.42 ± 0.13

[1] hours after denervation
[2] median value
n t = not tested

Bretylium and some related quaternary ammonium compounds also delay the start of the degeneration release of sympathetic transmitter (Benmiloud and Euler 1963 Langer 1966 and Lundberg 1969 and 1970 b) Bretylium seems to exert its delaying action at the distal parts of the neurons and it has a stronger delaying effect when given around the time of the expected start of the degeneration contraction of the rat periorbital smooth muscle, *e g* at 12 hrs after the denervation, than if injected earlier (Lundberg 1970 a) Colchicine, on the other hand was effective only when given early which points to a mode of action different from that of bretylium In order to delay the degeneration release colchicine probably needs to cover a longer part of the time interval between the axotomy and the expected onset of the degeneration contraction, *i e* the period during which the hypothetical "information is transported along the axon Hence, the tempting explanation of the present results is that the colchicine-induced delay of the start of the degeneration contraction is due to decreased rapidity of intra-axonal flow of some essential matter This would cause a delay of the wave of 'axotomy information" transported along the neuron

This study was supported by a grant from Magnus Bergvalls Stiftelse and by grant K 69 14X 733 04C to professor E Bárány from the Swedish Medical Research Council

References

BENMILOUD M and L S VON EULER Effects of bretylium reserpine guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland *Acta physiol scand* 1963 *59* 34—42

BORISY G G and E W TAYLOR The mechanism of action of colchicine *J cell Biol* 1967 *34* 525—533

DAHLSTROM A Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat *Europ J Pharmacol* 1968 *5* 111—113

LANGER S Z The degeneration contraction of the nictitating membrane in the unanesthetized cat *J Pharmacol exp Ther* 1966 *151* 66—72

KARLSSON J O and J SJOSTRAND The effect of colchicine on the axonal transport of protein in the optic nerve and tract of the rabbit *Brain Research* 1969 *13* 617—619

KREUTZBERG G Neuronal dynamics and axonal flow IV Blockade of intra axonal enzyme transport by colchicine *Proc nat Acad Sci* (Wash) 1969 *62* 722—725

LUNDBERG D Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat *Acta physiol scand* 1969 *75* 415—426

LUNDBERG D Bretylium and the degeneration contraction of the sympathetically innervated periorbital smooth muscle in the rat *Acta physiol scand* 1970 a *79* 411—427

LUNDBERG D Effects of some adrenergic neuron blockers related quaternary ammonium compounds and guanidine derivatives on degenerating adrenergic nerves in the conscious rat *Acta physiol scand* 1970 b *80* 323—344

Acta physiol. scand. 1970. 80. 433—435
From the Department of Zoophysiology, Göteborg, Sweden

Metabolism of Subcutaneously Injected ^{14}C-Histamine in Myxine Glutinosa

By

BJÖRN HOLSTEIN

Received 26 March 1970

Abstract

HOLSTEIN, B *Metabolism of subcutaneously injected ^{14}C-histamine in Myxine glutinosa* Acta physiol scand 1970 *80* 433—435

The recovery of total radioactivity, ^{14}C histamine and ^{14}C
Myxine glutinosa was determined after s c injection o
the same parameters were determined in single organs
ly 10 days after administration 71 % was recovered
methylhistamine amounted to 44 % and 1 % respectively of the given dose The liver was the most efficient organ in extracting radioactivity

Histamine is said to participate in many physiological processes and in mammals specific routes for its inactivation exists The most important metabolic pathways, elucidated mainly by experiments in mammals, are oxidative deamination and ring-N-methylation This communication deals with ^{14}C-histamine catabolism and elimination after subcutaneous administration to the cyclostome *Myxine glutinosa* The animals weighing 15—23 g, were also scanned for *in vitro* diamine oxidase (DAO) activity using putrescine (1 4-diaminobutane) as model substrate (Okuyama and Kobayashi 1961) *Myxine* were supplied by the Kristineberg Zoological station

After ^{14}C histamine administration, total radioactivity, ^{14}C histamine and ^{14}C-methylhistamine were assayed according to the method of Snyder *et al* (1964) Counting rates were transformed to dpm/g wet weight and results are presented as per cent of injected radioactivity in Fig 1 In experiments with single tissues, recovery of total radioactivity is given as per cent of injected dose, 100 % equalling $\frac{\text{dpm injected}}{\text{total weight of animal}}$ (dpm/g) Thus a recovery exceeding 100 % in a tissue means that radioactivity has been concentrated in that tissue ^{14}C histamine and ^{14}C methylhistamine appear in the table as per cent of total radioactivity recovered (Table I)

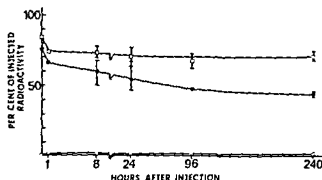


Fig 1 Recovery of total radioactivity (□), ^{14}C histamine (●) and ^{14}C methylhistamine (O) after s c injection of 0.5 ml saline containing 3.2 µg ^{14}C histamine, 1.0 µCi Values are the mean of 2—4 animals Vertical bars represent range

Results and discussion

There is a small decrease in total radioactivity during the first hour then the activity remains essentially constant for the time studied, which is, 10 days The ^{14}C-histamine content decreases remarkably slowly Methylhistamine is an unimportant metabolite, never exceeding 2 % of the injected dose The slow decrease of histamine is astonishing as DAO activity was found in both liver and intestine but may be explained by the findings of Blaschko *et al* (1969), a liver preparation of *Myxine glutinosa* attacked short-chain aliphatic diamines but not histamine It is unlikely that the subcutaneous route used for injection can explain the small elimination rate Injections were made in the great venous lacunae, thus resembling an i v infusion

Exogenous and endogenous histamine have been shown to rapidly equilibrate at least in some mammalian tissues (Johnson *et al* 1966 Beaven *et al* 1967) If such equilibration takes place in this species, the result indicates an extremely slow turnover rate of the amine

The disposition of radioactivity in single tissues was examined 24 hrs after ^{14}C-histamine injection the results appear in Table I In mammals, when injected i v

Table I *Recovery of total radioactivity ^{14}C-histamine and ^{14}C-methylhistamine Mean of 3 animals after s c injection of 3.2 µg ^{14}C-histamine, 0.5 µCi Animals frozen 24 hrs after injection*

tissue	total radioactivity % recovery[1]	^{14}C-histamine % of total activity	^{14}C-methylhistamine % of total activity
blood	127	70	1.8
bile	93[2]	22[2]	1.3[2]
intestine	48	33	2.8
liver	197	71	2.0
muscle	41	73	2.0
skin	61	60	3.4
brain	86		—

[1] $100\,\% = \dfrac{\text{dpm injected}}{\text{total weight of animal}}$

[2] Pooled from the 3 animals.

histamine is rapidly cleared from the blood (Rose and Browne 1938, Halpern *et al* 1959), the uptake of histamine in tissues has been suggested as an important mechanism for that This is obviously not the case in the slimehag, 24 hrs after injection the blood still contained 127 % of injected radioactivity, exceeded only by the liver, which was the most efficient organ in extracting radioactivity The histamine part of total radioactivity is generally high and the methylhistamine fraction very small The findings show that *Myxine* possesses no efficient system to eliminate histamine, or to render it pharmacologically inactive This questions the physiological importance of histamine in this animal representing a stomach less stage in evolution The histamine content in the digestive tract is low, resembling that of teleosts with reduced stomachs (Reite 1965, 1969a) When investigating effects of histamine on vascular smooth muscle Reite (1969b) found only weak responses in *Myxine* and they were considered as unspecific effects, "mediated through stimulation of β-adrenergic receptor mechanisms

References

BEAVEN M A Z HORAKOVA H
of histamine in rat gastric mucc

BLASCHKO H, M C BOADLE and
J Physiol (Lond) 1969 *204*

HALPERN B N TH NEVEU and C W M WILSON The distribution and fate of radioactive histamine in the rat. *J Physiol* (Lond) 1959 *147* 437—449

JOHNSON H L, M A BEAVEN F ERJAVEC and B B BRODIE, Selective labeling and release of non mast cell histamine *Life Sci* 1966 *5* 115—123

OKUYAMA T and Y KOBAYASHI Determination of diamine oxidase activity by liquid scintillation counting *Arch Biochem* 1961 *95* 242—250

REITE O B A phylogenetical approach to the functional significance of tissue mast cell histamine *Nature* (Lond) 1965 *206* 1334—1336

REITE O t cell histamine stores of the digestive tract *ntia* (Basel) 1969a *25* 276—277

REITE C responses to histamine and 5 hydroxy tryptar *Acta physiol scand* 1969b *75* 221—239

ROSE B and J S L BROWNE The distribution and rate of disappearance of intravenously injected histamine in the rat *Amer J Physiol* 1938 *124* 412—420

SNYDER S H J AXELROD and H BAUER The fate of C^{14} histamine in animal tissues *J Pharmacol exp Ther* 1964 *144* 373—379

Acta physiol scand 1970 80 436—442

From the Second Department of Pathology University of Helsinki Finland

The Response of RNA Synthesis to Mitotic Regulation in the Erythron

By

E Kivilaakso

Received 2 April 1970

Abstract

Kivilaakso E *The response of RNA synthesis to mitotic regulation in the erythron* Acta physiol scand 1970 80 436—442

factors affect RNA sy[illegible] [illegible] tion of the [illegible] ong inhibi[illegible] [illegible] lation by [illegible] mpensation by means of polycythemic target bone marrow on which the erythrocytic chalone had only a negligible effect The effect of the mitotic stimulator on ^{3}H uridine incorporation in the cells was invariably a stimulation which showed a slight tendency to increase with incubation time The results demonstrate that the effects of the mitotic regulatory factors are not restricted to DNA metabolism alone but can also be elicited within other metabolic processes in the cell

The maintenance of mitotic homeostasis in the erythron seems to be achieved by means of a dual control mechanism which regulates the balance between erythrocyte production and demand (Kivilaakso and Rytomaa 1970 a b Kivilaakso 1970) In normal steady state conditions the proliferation of the erythrocyte precursors is controlled by a humoral mitotic inhibitor the erythrocytic chalone as a negative feedback and in terms of the number of circulating red cells In response to an acute demand for erythrocytes a tissue specific mitotic stimulator becomes the dominant factor in rat serum

So far the effects of these regulatory factors have been studied in terms of DNA metabolism The present study describes some of their effects on RNA synthesis in rat bone marrow cells *in vitro*

Material and methods

Preparation of the regulatory factors

The mitotic inhibitor erythrocytic chalone was prepared in the same way as described before (Kivilaakso and Rytomaa 1970b)

[illegible]

Bioassay technique

The assay technique was the same as described in the preceding paper (Kivilaakso 1970) The composition of the final culture medium was Hanks BSS supplemented with 8 % old" (chalone free) rat serum 1 μCi/ml ^{3}H uridine (sp ac 29 6 Ci/m mole The Radiochemical Centre Amersham England) and 30 % of the crude preparation containing erythrocytic chalone or 60 % of the crude preparation containing mitotic stimulator

Polycythemic bone marrow

In some experiments polycythemic bone marrow rather than the normal tissue was used as the target tissue

The recipient rats received 3 ml of heparinized packed homologous RBC suspension in an iv injection in the v dorsalis penis on three consecutive days This treatment increased the RBC count of the recipient rats by about 50 per cent The bone marrow was withdrawn 3—4 days later and used immediately as the target tissue in the *in vitro* assay At this time very few erythrocyte precursor cells could be identified in the stained bone marrow preparations

Results

In the test conditions employed 80—90 % of the total ^{3}H uridine radioactivity incorporated in the cells is present in the RNA fraction as judged from an ordinary PCA hydrolysis (Rytomaa 1969 Kivilaakso unpublished results) Consequently the total radioactivities measured from the cells can be used to indicate the relative activity of RNA synthesis during incubation

A Effects of the mitotic inhibitor (erythrocytic chalone) on RNA synthesis

Incorporation of ^{3}H uridine in normal bone marrow cells

The effect of the erythrocytic chalone on the incorporation of ^{3}H uridine in normal bone marrow cells was examined in 8 independent experiments (192 individual cultures) A typical time course curve of the accumulation of ^{3}H uridine in the cells is shown in Fig 1

The results indicated that the primary effect of the erythrocytic chalone was to inhibit the incorporation of ^{3}H uridine in normal rat bone marrow cells Analysis of variance carried out on the raw data in each individual experiment suggested that a significant interaction may also exist between the material tested and the incubation time since in 5 of the 8 expts the interaction effect proved to be significant at the level $P \leqslant 0.05$ (see also Fig 1) The biological interpretation of this effect would be that the erythrocytic chalone influences the relative rate of RNA synthesis in the cells during incubation

In order to examine this interaction effect more thoroughly the results of the different experiments were scaled to a constant level (the mean of the experimental

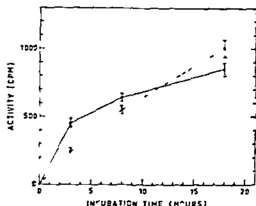


Fig 1

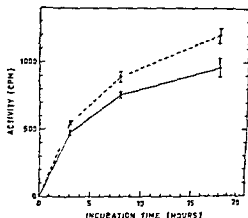


Fig 2

Fig 1 The effect of the erythrocytic chalone on the cumulative incorporation of ^3H uridine in normal rat bone marrow cells *in vitro* Mean ± SE of 4 experimental (○------○) and 4 control (●———●) cultures is presented at each incubation time The activities are expressed as total incorporation of ^3H uridine per culture In this experiment (expt II in Table I) the secondary stimulation in RNA synthesis is well seen

Fig 2 The effect of the erythrocytic mitotic stimulator on the cumulative incorporation of ^3H uridine in normal rat bone marrow cells *in vitro* Mean ± SE of 4 experimental (○- --- --○) and 4 control (●———● cultures is presented at each incubation time (expt. II in Table III) The activities are expressed as total incorporation of ^3H uridine per culture

group divided by the mean of the control group, respectively) before they were pooled The relative values expressed as percentage deviations from the controls are shown in Table I

It is seen that the erythrocytic chalone first caused a strong and statistically significant inhibition ($P<0\ 01$ Student s t-test) which tended to diminish ($P\sim0\ 025$, analysis of variance) during prolonged incubation It is thus apparent that the initial inhibition caused by the erythrocytic chalone is reversible, and may actually be followed by a real stimulation in the rate of the precursor uptake in the cells

Tissue specificity

Although the reversibility of the inhibition seems to rule out the possibility of any actual toxicity the results obtained so far do not provide unequivocal evidence that the effects are due to the regulatory influence of physiological feedback factors The best criterion of a physiological regulatory effect would be the demonstration of tissue specificity since the regulatory substance involved chalone, is tissue-specific by definition.

Tissue specificity was attempted to establish indirectly by using polycythemic bone marrow as the target tissue because it is depleted of most of the erythrocyte precursor cells The results are presented in Table II

TABLE I Effect of the erythrocytic chalone on the incorporation of ^{3}H uridine into normal rat bone marrow cells *in vitro*

Relative effect (% deviation from the control)			
	Incubation time		
Experiment	3 hrs	8 hrs	18 hrs
I	—41 6	+ 1 0	+53 7*
II	—46 8	—24 1	+17 0*
III	—10 2	+11 7	+52 3*
IV	—41 8	—37 2	—20 6
V	—33 8	+20 8	+45 1*
VI	—39 3	—42 4	—44 0
VII	—27 3	—37 2	—38 0
VIII	—15 1	—34 8	+26 9*
mean	—32 0 %	—17 8 %	+11 6 %

* Significant interaction effect ($P < 0\ 05$) between the material tested and the incubation time in an analysis of variance carried out on the raw data

TABLE II Effect of the erythrocytic chalone on the incorporation of ^{3}H uridine in polycythemic rat bone marrow cells *in vitro*

Relative effect (% deviation from the control)			
	Incubation time		
Experiment no	3 hrs	8 hrs	18 hrs
I	+ 1 5	+13 5	— 4 7
II	— 6 1	+12 6	+ 9 1
III	— 5 0	± 0 0	—14 4
IV	—11 2	—24 4	—19 2
V	—25 7	+ 1 5	+15 8
VI	—27 5	— 5 3	+ 5 3
mean	—12 3 %	— 0 4 %	— 1 4 %

TABLE III Effect of the erythrocytic mitotic stimulator on ^{3}H uridine incorporation by normal rat bone marrow cells *in vitro*

Relative effect (% deviation from the control)			
	Incubation time		
Experiment no	3 hrs	8 hrs	18 hrs
I	+ 5 2	+16 5	+23 6
II	+14 3	+17 2	+25 3
III	+26 8	+47 3	+23 6
IV	+ 9 7	+11 8	+18 8
mean	+14 0 %	+23 2 %	+22 8 %

It is seen that the chalone preparation now had only a slight inhibitory effect at the initial phase of incubation and that there was no statistical difference between the relative effects at different incubation times (analysis of variance $F<1$) In contrast to this, between the 3 hr effects obtained with normal and polycythemic target bone marrows there was a significant difference ($P\sim0\,01$, Wilcoxon s test) These results suggest that the erythrocyte precursor cells are the target in the bone marrow on which the erythrocytic chalone exerts its effect

B *Effects of the mitotic stimulator on RNA synthesis*

The effect of the mitotic stimulator on RNA synthesis in normal rat bone marrow cells was studied in 4 independent experiments (96 individual cultures) (Table III) A typical time course curve of the cumulative incorporation of ^{3}H uridine into the cells is shown in Fig 2

The results demonstrate that the material tested invariably stimulated accumulation of the label in the cells It is also seen that in three of the four experiments there was a slight tendency for stimulation to increase with incubation time although this tendency was not statistically significant (analysis of variance, $F<1$) The average stimulations obtained at different incubation times were 14 0, 23 2 and 22 8 % respectively

Discussion

The primary effect of the erythrocytic chalone on RNA synthesis was a rapid and strong inhibition It is clear that in *in vitro* assay conditions several sources of error may be present and this often makes it difficult to distinguish real physiological inhibitions from various unspecific and/or toxic disturbances In the present study the possibility of such disturbances was ruled out by the following findings (i) the inhibition observed in RNA synthesis was completely reversible (ii) the effects of the erythrocytic chalone did not involve bone marrow cells in general but showed considerable target tissue specificity (iii) similar erythrocytic chalone preparations have not exhibited toxic properties in earlier studies (Kivilaakso and Rytömaa 1970b)

Current experimental data suggested that the initiation of DNA duplication in mammalian cells requires the transient antecedent synthesis of a specific DNA dependent (actinomycin D sensitive) RNA (Lieberman *et al* 1963 Baserga *et al* 1965 Taylor 1965 Mueller and Kajiwara 1966) Although the primary function of the chalone is presumably to control some metabolic step prior to the initiation of DNA synthesis it is clear that it will be impossible to demonstrate inhibition of the synthesis of any particular type of RNA which will be present in amounts far too small to be detected with the present assay techniques It is thus concluded that the inhibitory effect observed is merely the consequence of a reduction in the number of erythrocyte precursor cells entering the generative cell cycle (Kivilaakso and Rytömaa 1970b)

The initial depression of RNA synthesis was followed after a few hours by a secondary stimulation which led to complete compensation of the cumulative incorporation of ^{3}H uridine in the cells. The biological basis for this "recovery" is not entirely clear and there may be several causes for it.

One possibility is that the stimulation is merely a compensatory reaction to the initial depression since it has been demonstrated (Rytomaa and Kiviniemi 1968) that the (granulocytic) chalone is inactivated/utilized within a few hours *in vitro*. However a direct stimulatory action of the erythrocytic chalone may also be involved. On theoretical grounds it has been deduced that the primary action of chalone may actually be to promote cellular maturation rather than to inhibit mitosis, the prevention of mitosis being only a secondary outcome of this action (Bullough and Rytomaa 1965). If so, one would expect the activity of those metabolic processes which underlie cellular maturation to increase.

It is evident that the messenger RNA governing haemaglobin synthesis in erythrocytic precursor cells is relatively stable (Grasso *et al* 1963, Orlic *et al* 1968). The same conclusion may also be valid for messenger RNA governing other maturative processes since in erythrocytic cells most of the RNA synthesis is limited to the earliest cell forms (Grasso *et al* 1963). On the other hand it is logical to assume that the messenger RNA which is involved in the initiation and maintenance of the generative cell cycle must have a relatively rapid turnover. Consequently the action of the erythrocytic chalone may be characterized by promotion of the synthesis of stable RNA but this promotion is quantitatively outweighed in the initial phase of incubation by the inhibition of the synthesis of short lived RNA.

The effect of the mitotic stimulator on RNA synthesis was invariably stimulation which had a slight tendency to increase with incubation time. It is obvious that the crude preparation employed in this study still contains some erythropoietin, for as pointed out in the previous study (Kivilaakso 1970) erythropoietin cannot be separated from the mitotic stimulator by means of the filtration technique employed. (The two factors may even be identical as was emphasized in that paper.) Consequently the effects observed may have been due to this "impurity" in the preparations tested. It has been demonstrated earlier (Krantz and Goldwasser 1965) that erythropoietin does have a stimulatory action on RNA synthesis in bone marrow cells *in vitro* but that this stimulation is not perceptible for several hours after the start of incubation. With respect to the present results three conclusions can thus be made: i) the initial stimulation of RNA synthesis is obviously not dependent on erythropoietin but rather is a secondary outcome of the entry of additional resting normoblasts into the generative cell cycle, (ii) erythropoietin may be at least partly responsible for the tendency of the stimulation to increase with incubation time, (iii) possible secondary or "delayed" responses in RNA synthesis will be masked by the effects of erythropoietin.

It was concluded in previous studies (Kivilaakso and Rytomaa 1970a, 1970b, Kivilaakso 1970) that the basic actions of the mitotic regulatory factors are to promote and prevent the entry of the erythrocyte precursor cells into the generative cell c

The present results support this conclusion by showing that the influences of the factors are not restricted to DNA metabolism alone, but that they can also be demonstrated in other metabolic processes in the cell

References

BASERGA, R, R ESTENSEN and R O PETERSEN, Inhibition of DNA synthesis in Ehrlich ascites cells by actinomycin D II The presynthetic block in the cell cycle *Proc nat Acad Sci* (Wash) 1965 *54* 1141—1148

BULLOUGH, W S and T RYTOMAA, Mitotic homeostasis *Nature* (Lond) 1965 *205* 573—578

GRASSO, J A, J W WOODARD and H SWIFT, Cytochemical studies on nucleic acids and proteins in erythrocytic development *Proc nat Acad Sci* (Wash) 1963 *50* 134—140

KIVILAAKSO, E, *In vitro* assay of the stimulatory effect of hypoxic serum on cellular proliferation in the erythron *Acta physiol scand* 1970 *80* 412—419

KIVILAAKSO, E and T RYTOMAA, The effect of polycythaemic serum on the proliferation of rat bone marrow cells *in vitro* *Cell Tissue Kinet* (In press) a

KIVILAAKSO, E and T RYTOMAA Erythrocytic chalone, a tissue-specific inhibitor of cell proliferation in the erythron *Cell Tissue Kinet* (In press) b

KRANTZ, S B and E GOLDWASSER, On the mechanism of erythropoietin induced differentiation II The effect on RNA synthesis *Biochim biophys Acta* (Amst) 1965 *103* 325—332

LIEBERMAN, I, R ABRAMS and P OVE, Changes in the metabolism of RNA preceding the synthesis of DNA in mammalian cells cultured from the animal *J biol Chem* 1963 *238* 2141—2149

MUELLER G C and K KAJIWARA, Actinomycin D and p-fluorophenylalanine, inhibitors of nuclear replication in the HeLa cells *Biochim biophys Acta* 1966 *119* 557—565

ORLIC, D, A S GORDON and J A G RHODIN, Ultrastructural and autoradiographic studies of erythropoietin-induced red cell production *Ann N York Acad Sci* 1968 *149* 198—216

RYTOMAA, T, Granulocytic chalone and antichalone *In Vitro* 1969 *4* 47—58

RYTÖMAA, T and K KIVINIEMI, Control of granulocyte production II Mode of action of chalone and antichalone *Cell Tissue Kinet* 1968 *1* 341—350

TAYLOR E W, Control of DNA synthesis in mammalian cells in culture *Exp Cell Res* 1965 *40* 316—332

Acta physiol. scand. 1970. 80. 443—449

From the Institute of Physiology, University of Oslo, Norway

Inhibitory Control of the Abdominal Stretch Receptors of the Crayfish

II. Reflex Input, Segmental Distribution, and Output Relations

By

J K S JANSEN, A NJÅ and L WALLOE

Received 3 April 1970

Abstract

JANSEN, J K S, A NJÅ and L WALLOE *Inhibitory control of the abdominal stretch receptors of the crayfish II Reflex input, segmental distribution, and output relations* Acta physiol scand 1970 *80* 443—449

The efferent activities in the thick and thin "accessory" axons in the dorsal nerve of the crayfish abdomen have been studied during reflex activation from the same and neighbouring segments The reflex [illegible] activated ipsilaterally while the thin accessory nerve predominantly participates in crossed reflexes

To assess the significance of the two accessory reflexes reported in the preceeding paper a determination of their distribution and their inputs is an obvious initial step Such data are presented in the present report. In some respects our observations differ from those published previously by Eckert (1961) and Fields *et al* (1967)

Methods

The experiments were performed on the isolated abdomen of fresh water crayfish (*Astacus fluviatilis*) The experimental conditions are presented in the preceeding paper (Jansen *et al* 1970) In experiments with adequate activation of the stretch receptors, the signals in the dorsal nerves were picked up "en passage' by suction electrodes. In other experiments the dorsal nerves were cut peripherally and the central end freed to the medial border of the slow extensor muscle The nerves could now be lifted into a layer of paraffine oil on pairs of platinum electrodes These were used for electrical stimulation of the nerves and recording of their activity An appreciable improvement in the resolution was obtained by recording in paraffine and this was important for the distinction between the smallest spikes

Results

The large inhibitory reflex spike can be activated not only by the slowly adapting stretch receptor (MRO_1), but also by the fast adapting stretch receptor (MRO_2) of the same and neighbouring segments. When both receptors are activated simultaneously, the efferent spike activity is greater than the reflex activity caused by any of the receptors alone. An example is shown in Fig. 1 which consists of simultaneous records from two ipsilateral dorsal nerves of neighbouring segments. A static stretch of the receptor muscle caused a steady afferent discharge from the slowly adapting receptor (A lower record). This afferent discharge activated the larger reflex spike in the neighbouring segment (A top record). When both the slowly and the rapidly adapting receptors were activated by vibration of the receptor muscles (B lower record) the frequency of the reflex spike was greater (B top record). This is in agreement with results previously reported by Eckert (1961a).

By stretch of the thin receptor muscle (RM_1) and vibration applied to the thick receptor muscle (RM_2) it proved possible to activate the MRO_1 and MRO_2 independently or in combination. The input—output relations of such reflexes are shown in Fig. 2. The frequency of the large inhibitory reflex spike is plotted against the input frequencies from the MRO_2 (+), the MRO_1 (×) and both receptors (0) respectively. The figure shows that the output frequency seems to increase monotonically with input frequency for all these three kinds of input. This was a regular finding but the gain was often larger than shown in this figure, sometimes as high as one for the slowly adapting receptor alone (see Fig. 1 of preceeding paper).

The reflex spikes can also be activated by electrical stimulation of the dorsal nerve. Fig. 3 shows the impulse frequency of the large reflex spike as a function of the amplitude of the stimulus current pulse applied in the neighbouring segment. The output frequency increased in two steps with increasing stimulus amplitude indicating that only two afferent axons participate in the reflex. The two MRO afferent fibres are the largest and perhaps the only afferents in the dorsal nerve (Alexandrowicz 1967). The reflex activation of the large accessory spike was elicited at a stimulus intensity close to the threshold of the largest axons of the nerve. The MRO_2 axon is the thickest of the two afferents and the first step of the intensity response curve probably represents activation of this axon only. This agrees with

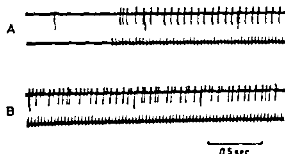


Fig. 1. Convergence of MRO_1 and MRO_2 signals on the thick accessory neurone.
A. Accessory reflex of third segment (top record) elicited by stretch activation of ipsilateral MRO_1 of second segment (lower record).
B. Same reflex. Increased accessory fibre output by simultaneous activation of MRO_1 and MRO_2 (lower record). MRO_1 frequencies are equal in A and B.

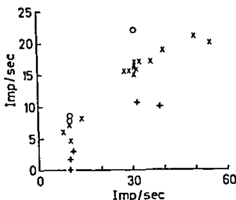


Fig 2

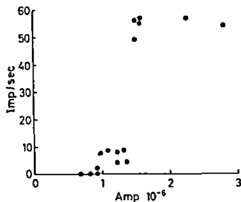


Fig 3

Fig 3 Plot of the output frequency (thick accessory fiber of third segment) as a function of the intensity of the electrical stimulation, applied at a constant frequency of 57/sec to the ipsilateral dorsal nerve of fourth segment

Eckert's (1961a) observation that the reflex output was often rather small when activating MRO_2 only In many preparations the threshold difference between the two afferents was smaller than in the experiment of Fig 3 so that stimulation of the thickest fibre separately was often difficult at moderate and high stimulus repetition rates

Fig 4 gives a comparison of the effects of electrical and adequate mechanical activation of the receptors Activation of MRO_2 alone (+) gave only a weak reflex response The MRO_1 (×) gave a more powerful reflex output and the simultaneous activation of both receptors (0) gave some addition increase at a given input frequency Supramaximal electrical stimulation (●) of the nerve gave a closely comparable output which strongly suggests that the electrically elicited reflex is due to activation of the two MRO afferents On account of their greater convenience

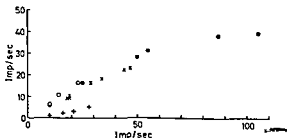


Fig 4 Comparison of reflex effects to electrical and adequate activation Abscissa and ordinates as in Fig 2 + Input from MRO_2 alone × Input from MRO_1 alone ○ Simultaneous activation of MRO_1 and MRO_2 ● Repetitive electrical stimulation at intensities above threshold for both afferent axons

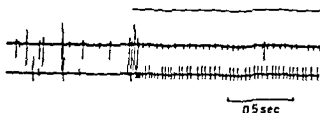


Fig 5 Accessory reflexes in ipsilateral and contralateral dorsal nerves Stimulation of left dorsal nerve of fourth segment at 90/sec indicated by line above records Top record Activity of right dorsal nerve of third segment Small spike reflexly activated Lower record Large spike activated ipsilaterally in third segment Amplifier gain five times higher in top record

electrically activated reflexes were mainly used for the study of the distribution of these effects among the different segments

As demonstrated in the preceeding paper both the thick and the thin accessory nerve may be reflexly activated by a stretch receptor input in the same and neighbouring segments The distribution of these reflexes are of obvious functional significance Fields *et al* (1967) found that the thick accessory nerve appeared to be more strongly activated in segments in front of that of the input We have found a certain difference in the distribution of the effects to the large and the small accessory nerve The large accessory nerve tended to be more consistently activated in ipsilateral neighbouring nerves while the small fibre activation was more prominent in contralateral nerves A typical example is presented in Fig 5 which consists of simultaneous records from two dorsal nerves The top record is from a nerve contralateral to that stimulated while the lower is from the ipsilateral neighbouring nerve It appears that a small spike was activated in the contralateral while a larger spike occurred in the ipsilateral nerve during the stimulation

However there is a fair amount of variation from preparation to preparation Sometimes a reflex might be absent between two neighbouring nerves even though we could demonstrate that the stretch receptor afferents of the one nerve and the accessory fibers of the other was intact by respectively their input effects or their activation in reflex connections with other dorsal nerves

Therefore in order to obtain a meaningful assessment of the distribution of these reflexes a series of five consecutive experiments were done in which all the reflex connections between the six dorsal nerves of the 2 3 and 4 abdominal segment were recorded Each nerve was stimulated by a standard input of 50 shocks/sec above the threshold for the stretch receptor afferents and the rate of firing of the accessory nerves was used as an index of the strength of reflex coupling to that particular nerve The large and the small accessory nerve spikes were unambiguously distinguished by their amplitudes The small spikes were less than one third of the size of the large one in all these five experiments

The results of these five experiments are summarized in Fig 6 The circles indicates the six dorsal nerves and the arrows indicate the observed reflex effects between these nerves A gives the observations for the large accessory fibre and B those for the small The number in each arrow gives the mean frequency of firing

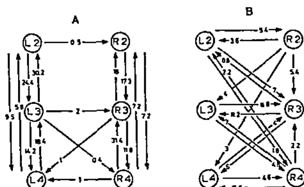


Fig 6 The distribution of the large spike reflex (A) and the small spike reflex (B) Data from five consecutive experiments The symbols enclosed in the circles represent the dorsal nerves The arrows connecting the circles represent observed reflex responses, and the numbers assigned to the arrows indicate the mean frequencies (imp/sec) obtained from the 5 expts The standard input was electrical stimulation of the dorsal nerves one at a time, at a frequency of 50/sec Note the predominance of large spikes to ipsilateral nerves and small spikes to contralateral nerves

of that particular reflex to the 50 shock/sec input and is thus our index of reflex coupling Low numbers of course indicate frequent absence of a particular connection Absence of an arrow indicate that no such reflex was seen in the series of five experiments It appears as our preliminary observations suggested that the activation of the large accessory nerve mainly occurred in ipsilateral nerves whereas the activation of the small accessory nerve was mainly seen in crossed reflexes Furthermore, Fig 6 supports Eckert s (1961a) finding that the reflex coupling is stronger to neighbouring than to more distant nerves In six experiments we have been able to compare the reflex output in the activated segment with that of the neighbouring segments and again we have confirmed Eckert (1961a) in finding no significant difference between the two The notion that the large accessory reflex is more powerful in an anterior than in a posterior direction (Fields *et al* 1967) is also supported by the present data The differences are however, small and just significant at a 0 05 level in the Wilcoxon test

It remains to be shown that the small spike activated in contralateral reflexes is indeed inhibitory to the stretch receptors This appears from Fig 7 The top record is an en passage registration from a dorsal nerve During electrical stimulation of the contralateral nerve the discharge rate of the slowly adapting receptor was



Fig 7 The inhibitory effect of the small reflex spike A "En passage record from an intact dorsal nerve during electrical stimulation of a contralateral dorsal nerve The slowly adapting receptor (large spike) is inhibited B Same reflex recorded in paraffine after distal section and liberation of the dorsal nerve A small unit is reflexly activated during the electrical stimulation of the contralateral nerve The straight line at the top indicates the duration of the stimulus period in both

reduced The resolution of the "en passage" record was, *however, insufficient to* reveal the small inhibitory spikes The nerve was therefore cut peripherally and its activity recorded in paraffine oil (lower record) During the same stimulus the reflexly activated small spike was now clearly apparent

Discussion

The most important finding of the present paper is the differential distribution of the two accessory reflexes Even though the difference was only partial, there was a clear dominance of the small fibre in the crossed and the large fibre in the ipsilateral reflexes (Fig 6) The significance of this is not possible to assess with our present knowledge Assuming that the abdominal stretch receptors are of importance for the control of body movements, it suggests, however, assymetries in the innervation of the abdominal muscles and the possibility of independent control of the slow extensor muscles on each side of an abdominal segment The usefulness of this is difficult to visualize in view of the commonly accepted view that the joint between the abdominal segments only operate around a single transverse axis It should be pointed out however, that the stretch receptor activation of the excitatory motoneurone of the slow extensor muscle appear to be confined to the ipsilateral side (Fields 1966)

Dismissing the possible importance of independent control of the slow extensor muscles on each side which is rather far fetched it is attractive to regard the reflex activation of the thin accessory fibre simply as an extension of the thick accessory nerve reflex to increase its distribution To the extent that there is overlap between the two reflexes, it will simply act to increase the 'gain' of that particular inhibitory feedback to the stretch receptors

Fields *et al* (1967) suggested that the accessory reflex might serve to coordinate the motor activity of different segments directionally The small difference in anterio-posterior distribution of the reflex found in the present experiments makes this suggestion less attractive It may therefore from the point of view of the accessory reflex be more useful to regard the entire abdomen as a unit consisting of segmental flexor and extensor muscles Then one is struck by the numerous analogies with the innervation of vertebrate skeletal muscles The MRO excitation of the excitatory motoneurone corresponds to a stretch reflex and the accessory reflex may be analogous with the recurrent inhibition of the motoneurones The interesting feature is that the accessory reflex is placed as a negative feedback on the input side instead of on the output side as is the Renshaw inhibition But on account of the loop delay it may quite possible act in the same manner as a high pass filter Quantitative determinations of the various loop gains and delay times are however required to establish *the usefulness of the analogy*

A second point deserves comment In his original description Eckert (1961a) suggested that the accessory reflex was mediated via one or more interneurones and this appears to have been accepted implicitly in subsequent investigations The reasons for Eckerts suggestion were however rather weak as he stated himself His main

observations in favour of interneurones were that the accessory reflex disappeared if the two connectives of a particular ganglion was sectioned and the decreasing effects or reflex inputs more than one segment away from the output However it is known that the stretch receptor afferents divide centrally and give rise to ascending and descending collaterals Assuming that these have collaterals in each ganglion establishing synaptic connections with the accessory neurones and that the efficiency of these connections decreases with increasing distance from the segment of entry the entire accessory reflex pattern might be explained The central delay of the accessory reflex some 20—30 msec may seem long for a monosynaptic connection. However equally long apparently monosynaptic delays have been described in invertebrates (Wachtel and Kandel 1967) The main observation in favour of a monosynaptic connection for the accessory reflex is the apparent simplicity of signal transfer None of the common characteristics of polysynaptic reflexes such as after discharges or synaptic failure during maintained activation have been observed Indeed the common finding of a one to one transmission in the accessory reflex (Jansen *et al* 1970 Fig 1) would require a very particular polysynaptic coupling and seems unlikely in view of the great economy of neuronal machinery found in invertebrates But the postulation of a monosynaptic connection for the accessory reflex must still be regarded only as a working hypothesis even if it is the most plausible one in view of present knowledge

References

ALEXANDROVICZ J S Receptor organs in the thoracic and abdominal muscles of crustacea *Biol Rev* 1967 *42* 288—326

ECKERT R. O Reflex relationships of the adbominal stretch receptors of the crayfish. I Feed back inhibition of the receptors *J cell comp Physiol* 1961a. *57* 149—169

ECKERT R. O Reflex relationships of the abdominal stretch repectors of the crayfish. II Stretch receptor involvement during the swimming reflex. *J cell comp Physiol* 1961b *57* 163—174

FIELDS H L W H EVOY and D KENNEDY Reflex role played by efferent control of an invertebrate stretch receptor *J Neurophysiol* 1967 *30* 859—874

JANSEN J K S A NJA and L WALLOE Inhibitory control of the abdominal stretch receptors of the crayfish I The existence of a double inhibitory feedback *Acta physiol scand* 1970 *80* 420—425

WACHTEL H and E R KANDEL A direct synaptic connection mediating both excitation and inhibition *Science* 1967 *158* 1206—1208

Acta physiol scand 1970 80 450—458

From the Department of Pharmacology, Karolinska Institutet Stockholm Sweden

Effects of Prostaglandin E_1 in Canine Subcutaneous Adipose Tissue in Situ[1]

By

BERTIL B FREDHOLM and SUNE ROSELL

Received 10 April 1970

Abstract

FREDHOLM, B B and S ROSELL. *Effects of prostaglandin E_1 in canine subcutaneous adipose tissue in situ* Acta physiol scand 1970 *80* 450—458

The effect of PGE_1 on the uptake of glucose and the release of FFA and glycerol before and after sympathetic nerve stimulation (4 cps) was investigated in perfused canine subcutaneous adipose tissue *in situ* Glucose uptake was significantly increased by PGE_1 at all concentrations used (5×10^{-10} to 7×10^{-7} M in blood) The effect of PGE_1 on the release of FFA and glycerol in unstimulated adipose tissue was inconsistent Increases as well as decreases were observed Lipolysis as measured by glycerol release induced by nerve stimulation was inhibited dose-dependently A 50 per cent inhibition was produced by approximately 1.2×10^{-7} M PGE_1 Stimulated FFA release was also inhibited but there was no clear dose response relationship It is concluded that PGE_1 has similar effects in canine subcutaneous adipose tissue with an intact blood supply as are known to be produced *in vitro*

The prostaglandins inhibit basal and catecholamine stimulated lipolysis tissue *in vitro* and decrease plasma free fatty acid (FFA) and glycerol levels in intact animals and man (see Bergstrom Carlson and Weeks 1968) Moreover Shaw and Ramwell (1968) have demonstrated that nerve stimulation releases prostaglandins from incubated rat epididymal fat pads This observation has raised the possibility that prostaglandins act as locally released feedback inhibitors of lipolysis in adipose tissue (Shaw and Ramwell 1968)

The present experiments were undertaken with this hypothesis as a background The capacity of PGE_1 to antagonize the lipolytic effect of nerve stimulation in canine adipose tissue *in situ* was determined The effect of PGE_1 on FFA release and glucose uptake was also investigated

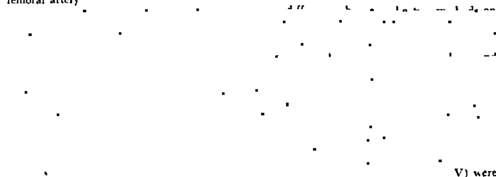
Materials and methods

The experiments were conducted on 27 fed female mongrel dogs weighing between 8 and 16 kg The dogs were anesthetized with 25 mg sodium pentobarbital per kg b w i v with supplement when necessary The subcutaneous adipose tissue (19—61 g mean 37 g) on the

[1] Part of these results have been presented in preliminary form (Fredholm and Rosell Acta physiol scand 1968 74 6 A)

right side of the abdominal midline was isolated from all surrounding tissues and perfused with blood from the same dog (Rosell 1966) according to either of two methods In 21 expts blood was drawn from the contralateral femoral artery, defibrinated and placed in a reservoir The tissue was perfused at a constant rate according to Renkin and Rosell (1962) The venous outflow passed a drop-counter to monitor the blood flow

In the remaining 6 expts heparin 2000 IU/kg b w was given i v A drop counter was inserted between the femoral artery on the same side as the adipose tissue and the artery to the adipose tissue The venous outflow from the adipose tissue was returned to the systemic circulation via the contralateral femoral vein Venous samples could be collected by means of a three way stop cock The systemic arterial pressure was measured from the contralateral femoral artery



V) were

l samples were taken from the reservoir or from the femoral artery After centrifugation aliquotes of plasma were taken for the analysis of glucose (using commercially obtainable glucose oxidase reagent Glox, Kabi Stockholm), glycerol (Laurell and Tibbling 1966) and FFA (Trout *et al* 1960)

The arterial levels of the metabolites varied between the dogs Thus arterial glycerol concentrations ranged between 0 06 and 0 45 mM arterial FFA levels between 0 08 and 0 74 mM and the arterial glucose levels between 1 3 and 5 4 mM There were no statistically significant differences between the free flow and the constant flow experiments

The net release or net uptake was calculated as the arteriovenous differences multiplied by the plasma flow per 100 g of adipose tissue There is no association between the arterial FFA and glycerol concentration and the rate of uptake or release of these substances (Fredholm 1970) Statistical analysis was performed according to Dixon and Massey (1957)

Results

Prostaglandin E_1 infusion The results of these experiments are given in Table I

Glycerol release The basal glycerol release rate was 0 24 ± 0 04 μmoles × min^{-1} × 100 g^{-1} (mean ± S E n = 16) The rate was increased by nerve stimulation, as is seen from Fig 1 to 3 By subtracting the basal release rate from that during and after stimulation the net amount of glycerol released by nerve stimulation was found to be *24 5 ± 5 5* μmoles × 100 g^{-1}

Administration of PGE_1 did not produce any significant changes in the rate of glycerol release in unstimulated adipose tissue Both increases (see Fig 3) and decreases (see Fig 1) were seen In practically all experiments the cessation of the PGE_1 infusion was followed by a transient increase in the glycerol release (see Fig 1—3)

The release of glycerol upon sympathetic nerve stimulation was inhibited by PGE_1 in a dose dependent fashion (Fig 4) Owing to the limited number of ex-

TABLE I Metabolic effects of PGE_1 in canine subcutaneous adipose tissue Glycerol and I
nerve stimulation is given as the net amount in μmoles/100 g released over basal rele
tissue minus μmoles FFA released/60 min/100 g tissue The glucose uptake is simila
t test for paired variates

Dog no	Perf	GLYCEROL RELEASE			
		control basal	stim	PGE treated basal	stim
PGE_1 (5—7 × 10 [illegible])					
2	C F	0.14	29.7	0.29	10.2
6	C F	0.45	25.9	0.23	2.8
10	C F	0.09		0.02	
17	F F	0.38		0.19	
Mean		*0.26*	*27.8*	*0.18*	*6.5*
PGE_1 (4—7 × 10 [illegible])					
1	C F	0.04	4.7	0.02	3.6
5	C F	0.33	42.7	0.60	26.8
9	C F	0.22	19.4	0.29	5.7
10	C F	0.30		0.31	
12	F F				
13	F F	0.06		0.03	
16	F F	0.04		0.06	
18	C F	0.36		0.45	
Mean		*0.19*	*22.3*	*0.25*	*12.0* p 0.05
PGE_1 (4—7 × 10 [illegible]					
2	C F	0.09	17.4	0.09	15.1
4	C F	0.08	18.0	0.35	16.4
7	C F	0.08	15.3	0.04	10.5
10	C F	0.72		0.38	
11	C F	0.16		0.72	
12	F F				
13	F F	0.11		0.10	
15	F F				
18	C F	0.25		0.15	
Mean		*0.21*	*16.9*	*0.26*	*14.0* p 0.05
PGE_1 (5—7 10 [illegible]					
3	C F	0.48	39.2	0.86	40.8
8	C F	0.49	31.8	0.44	25.2
9	C F	0.62	21.9	0.37	17.1
11	C F	0.03		0.18	
12	F F				
13	F F	0.12		0.10	
14	F F	0.04		0.02	
18	C F	0.32		0.40	
Mean		*0.31*	*31.0*	*0.31*	*27.7*

release during basal conditions are given as μmoles/min/100 g Glycerol and FFA release following Re esterification (μmoles hr/100 g) is here taken to mean 3 × μmoles glycerol released/60 min/100 g given as μmoles taken up/60 min/100 g tissue Statistical hypotheses were tested by Student's

FFA RELEASE				RE-ESTERIFICATION		GLUCOSE UPTAKE	
control basal	stim	PGE treated basal	stim	control	PGE treated	control	PGE treated
0 02	32 3	0 07	7 5	70	20	65	72
—0 22		—0 43		132	86	145	235
0 08		0 01		11	6	36	40
						73	106
—0 04	*32 3*	*—0 12*	*7 5*	*71*	*37*	*80*	*113* p < 0 05
0 19	3 0	—0 06	1 4	16	14	319	497
0 02	16 9	0 14	10 8	92	66		
—0 06	14 1	—0 02	4 3	150	54	254	255
0 15		0 13		18	13	45	60
						42	51
—0 13		0 30		12	0	43	38
						39	358
0 31		0 33		46	68	54	87
0 08	*11 3*	*0 14*	*5 5* p < 0 05	*56*	*36*	*114*	*192* p < 0 01
—0 10	14 0	0 16	8 3	71	46	99	114
—0 32	6 9	1 15	6 1	47	15	32	56
—0 22	49 1	—0 25	13 2	39	53	51	92
0 50		0 24		100	54	61	98
						186	231
						68	91
—0 01		—0 03		20	20	61	69
						64	59
—0 04		0 02		47	26	150	424
—0 03	*23 3*	*0 22*	*9 2* p ~ 0 05	*54*	*36* p < 0 05	*86*	*137* p < 0 01
0 11	7 9	0 04	3 3	135	178	92	143
0 13	3 2	0 04	2 1	132	97	91	173
						153	195
						93	208
						35	50
0 02		—0 02		22	19	52	41
						40	13
—0 09		—0 25		51	87	136	276
0 04	*5 6*	*—0 05* p < 0 02	*2 7*	*85*	*95*	*86*	*137* p < 0 01

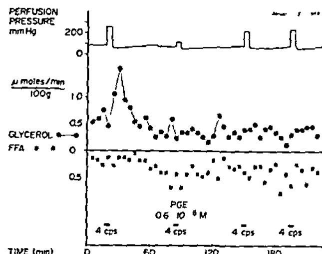


Fig 1 The effect of 6×10⁻⁷ M PGE_1 on perfusion pressure glycerol and FFA release rates 10 kg dog Anesthetized with Nembutal Adipose tissue (25 g) perfused with defibrinated blood (Hct 32 0) at a constant rate (6 2 ml/min 100 g) Afferent nerve to adipose tissue stimulated for 5 min at indicated times

periments the determination of the PGE_1 concentration producing 50 % inhibition of lipolysis (1.2×10^{-7} M) was subject to large errors

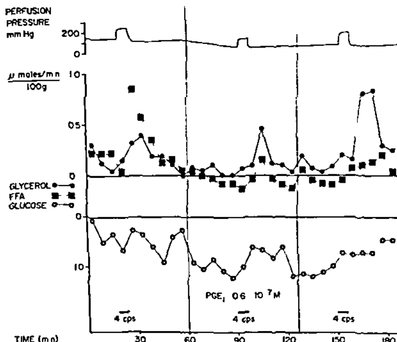
FFA release On the average 0 02 ± 0 08 μmoles × min⁻¹ × 100 g⁻¹ (n = 11) FFA were released during basal conditions Net uptake of FFA was seen as often as net release Stimulation of the nerves to the adipose tissue caused a release of FFA (16 4 ± 3 5 μmoles FFA per 100 g of tissue) The experiment illustrated in Fig 1 was the only exception to this rule

Only with the lowest concentration of PGE_1 was there a decreased basal release of FFA (Table I) With higher concentrations transient increases in FFA were occasionally seen (e g Fig 3) Transient increases in FFA release were also noticed upon the cessation of PGE_1 administration

PGE_1 inhibited stimulated FFA release (12—77 per cent) but no dose response relationship was found This is evident from Table I

Glucose uptake The rate of glucose uptake by adipose tissue was 1 38 ± 0 17 μmoles × min⁻¹ × 100 g⁻¹ (n = 17) and was apparently linearly dependent upon the arterial glucose concentration ($p < 0.01$) No statistically significant differences between the glucose uptake before during and after nerve stimulation were revealed As is shown in Table I PGE_1 at all concentrations investigated caused a marked increase in the glucose uptake No dose response relationship was noticed however

Re-esterification The values given in Table I under this heading were computed by multiplying the total net release of glycerol by three and subtracting the total net release of FFA The rationale behind this calculation is discussed by Steinberg and Vaughan (1965) With 5—7 × 10⁻¹⁰ M PGE_1 no change was seen With higher concentration of PGE_1 re-esterification was reduced to a variable degree respective to control (Table I)



F... and on ... Adipose ... ml/min/

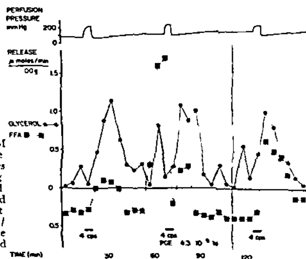


Fig 3 The effect of 4×10^{-9} M PGE_1 on perfusion pressure glycerol and FFA release rates (μmoles/min/100 g) Dog 12 kg Anesthetized with Nembutal Adipose tissue (45 g) perfused with defibrinated blood (Hct 31 5 at a constant rate (4 9 ml/min/100 g) Afferent nerve tissue stimulated for 5 min at indicated times

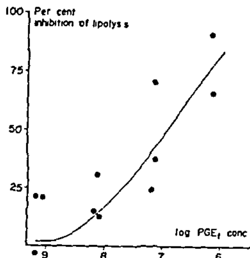


Fig 4 The relationship between the concentration of PGE_1 in arterial blood and the per cent inhibition of lipolysis evoked by nerve stimulation The regression line was calculated after transforming the data using log normal probability graph paper The regression was significant at the 2 per cent level Deviations from linearity were insignificant

Vascular reactions The vascular resistance was decreased in all experiments where the PGE_1 concentration was higher than 10^{-8} M and in 1 expt with 6×10^{-9} M PGE_1 *The threshold for this effect was therefore in the neighbourhood of* 10^{-8} M Upon nerve stimulation vascular resistance increased by 74 to 311 per cent (mean 180 per cent) This effect of nerve stimulation could not be counteracted by PGE_1 in concentrations below 10 M In 2 expts with 6×10^{-6} M vascular resistance following nerve stimulation increased by only 63 as compared to 190 per cent during the corresponding control stimulation The experiment illustrated in Fig 1 demonstrates that following the cessation of the PGE_1 infusion the vascular responses to nerve stimulation return to normal On the other hand the glycerol release upon nerve *stimulation did not return to normal even after more than 1 hr*

Injections of prostaglandin E_1 In 9 dogs PGE_1 (0 2—2000 ng) was injected i.a The effect on the lipolytic rate determined by the glycerol release and on FFA release was investigated As was seen with the infusion experiments the effect of PGE_1 administration on lipolysis and FFA release during basal condition was very variable Thus both transient increases and decreases in the release rates were seen at all doses used

Discussion

The effects of prostaglandins have been studied extensively both *in vitro* and in intact animals and man (see Bergstrom Carlson and Weeks 1968) It has become evident that they especially PGE_1 are potent inhibitors of catecholamine stimulated lipolysis Since sympathetic nerve stimulation is more effective for inducing lipolysis than infused catecholamines at least in canine subcutaneous adipose tissue (Ballard Cobb and Rosell 1970) it was considered of interest to study the effect of PGE_1 on the vascular and metabolic events following nerve stimulation The vascular effects

of PGE_1 have been reported elsewhere (Fredholm Öberg and Rosell 1970) The effect of PGE_1 *in vitro* on lipolysis induced by nerve stimulation (25 cps) was studied in rat epididymal fat pads by Berti and Usardi (1964) They found that 1 ng/ml in the medium (about 3×10^{-9} M) significantly reduced the outflow of FFA and glycerol upon nerve stimulation However in order to evaluate the physiological significance of prostaglandins in adipose tissue during stimulation it is desirable to provide the tissue with an intact blood supply and furthermore to limit the impulse frequency in the sympathetic nerves so that it does not exceed 6—8 cps (see Folkow 1952) Accordingly we have used 4 cps in the present study This frequency also produces a maximal rate of FFA release (Rosell 1966)

The data obtained are in agreement with the earlier *in vitro* studies Lipolysis evoked by nerve stimulation was reduced to one half by a blood level of about $1\ 2\times 10^{-7}$ M PGE_1 The sensitivity of adipose tissue to prostaglandins is thus high enough to consider the possibility that they are physiologically important inhibitors of lipolysis

With an experimental technique similar to that used by Berti and Usardi (1964) Shaw and Ramwell (1968) have shown that nerve stimulation evoked the release of prostaglandins from rat epididymal fat pads Small amounts of prostaglandin like material were also found by us in venous blood from canine subcutaneous adipose tissue during and after nerve stimulation (4—10 cps) but there was no increase in the tissue content following nerve stimulation for 20 min (Fredholm Rosell and Strandberg 1970) It should be pointed out however that adipose tissue of the swine has a high prostaglandin metabolizing activity (Änggård Larsson and Samuelsson 1970) and that only 5 to 20 per cent of PGE_1 infused to canine subcutaneous adipose tissue was recovered in the venous blood (Fredholm Rosell and Strandberg 1970) One must therefore consider the possibility that local prostaglandin concentrations may be considerably higher than those found in tissue or venous blood

Recently adrenergic α receptor blockade was found to potentiate the lipolytic response to nerve stimulation (Fredholm and Rosell 1968) probably because of changes in the vascular response after α blockade (Linde and Rosell 1970 Fredholm Öberg and Rosell 1970 Fredholm and Rosell 1970) In addition it is possible that lipolysis *per se* was inhibited by adrenergic α receptor stimulation and that augmented fatty acid release following α blockade could be due to selective removal of inhibition by prostaglandin as suggested by Bergstrom Carlson and Weeks (1968) However Shaw and Ramwell (1968) found that prostaglandin efflux was associated with lipolysis which seems to be regulated by β receptor stimulation Moreover there was no indication of an inhibition of prostaglandin efflux by dihydroergotamine (Fredholm Rosell and Strandberg 1970) and recent evidence from this laboratory does not support the hypothesis that α receptor stimulation inhibits lipolysis (Fredholm 1970 Fredholm and Karlsson 1970)

It is thus at present difficult to clarify the part played by prostaglandins in the lipolytic response following nerve stimulation in subcutaneous adipose tissue of the dog It is also difficult to ascribe any of the [illegible]

tion to prostaglandins (Fredholm Öberg and Rosell 1970) It should be pointed out however, that the present experiments were designed to study acute effects and they give no indication of a possible role played by prostaglandins in the long term regulation of adipose tissue metabolism

These studies were supported by grants from the Medical Research Council (B69 14X 731-04B B68-40X 2553-01, B69-40X 2553-02) Karolinska Institutet and the Swedish Society for Medical Research

References

Änggård E C Larsson and B Samuelsson The distribution of 15 hydroxy prostaglandin dehydrogenase and prostaglandin Δ^{13} reductase in different tissues of the swine *Acta physiol scand* 1971 In press

Ballard K C A Cobb and S Rosell Vascular and lipolytic responses in canine subcutaneous adipose tissue following infusion of catecholamines *Acta physiol scand* 1971 In press

Bergström S, L A Carlson and J R Weeks The prostaglandins A family of biologically active lipids *Pharmacol Rev* 1968 20 1—48

Berti F and M M Usardi Investigations on a new inhibitor of free fatty acid mobilization *G Arterioscler* 1964 2 261—265

Dixon W J and F J Massey Jr *Introduction to statistical analysis* 2nd ed Mc Graw Hill New York 1957

Folkow B Impulse frequency in sympathetic vasomotor nerves correlated to the release and elimination of the transmitter *Acta physiol scand* 1952 25 49—76

Fredholm B B Studies on the sympathetic regulation of circulation and metabolism in isolated canine subcutaneous adipose tissue *Acta physiol scand* 1970 Suppl 354

Fredholm B B and J Karlsson Metabolic effects of prolonged sympathetic nerve stimulation in canine subcutaneous adipose tissue *Acta physiol scand* 1970 80 567 576

Fredholm B B B Öberg and S Rosell The effect of vasoactive drugs on circulation in canine subcutaneous adipose tissue by nerve stimulation 1970 *Acta physiol scand* 1970 79 364—374

Fredholm B B and S Rosell Effect of adrenergic blocking agents on lipid mobilization from canine subcutaneous adipose tissue after sympathetic nerve stimulation *J Pharmacol exp Ther* 1968 159 1—7

Fredholm B B and S Rosell Fate of ³H noradrenaline in canine subcutaneous adipose tissue 1970 *Acta physiol scand* 1970 80 404—411

Fredholm B B S Rosell and K Strandberg Release of prostaglandin like material from canine subcutaneous adipose tissue by nerve stimulation 1970 79 18A—19A

Laurell S and G Tibbling An enzymatic fluorometric micromethod for the determination of glycerol *Clin chim acta* 1966 13 317—322

Linde B and S Rosell To be published

Ngai S H S Rosell and L Wallenberg Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs *Acta physiol scand* 1966 68 397 403

Renkin E and S Rosell The influence of sympathetic adrenergic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in skeletal muscle *Acta physiol scand* 1962 54 223 240

Rosell S Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation *Acta physiol scand* 1966 67 343 351

Shaw J E and P W Ramwell Release of prostaglandin from rat epididymal fat pad on nervous and hormonal stimulation *J biol Chem* 1968 243 1498 1503

Steinberg D and M Vaughan Release of free fatty acid from adipose tissue in vitro in relation to rates of triglyceride synthesis and degradation In *Handbook of Physiology Section 5 Adipose Tissue* eds A E Renold and G F Cahill Jr pp 335 347 Williams and Wilkins Co Baltimore 1965

Trout, D L Estes H and S J Friedberg Titration of free fatty acids of plasma A study of current methods and a new modification *J Lipid Res* 1960 1 199 202

Acta physiol scand 1970 80 459—469

From the Institute of Physiology University of Oslo Norway

Reduction in Pulmonary Blood Volume after a Blood Loss

By

PETTER AARSETH

Received 23 April 19 0

Abstract

AARSETH P *Reduction in pulmonary blood volume after a blood loss* Acta physiol scand 1970 *80* 459—469

There is little informat on about the possible role of the pulmonary vascular bed as a variable blood depot. An evaluation of the degree of blood mobilization from this vascular compartment after a blood loss has therefore been attempted Rats were given labelled erythrocytes and al

5 while the muscle and liver blood contents were reduced by about 10 % only This dif

d cat ng that there had been a reduction also in lung tissue water

The volume of blood in the lungs is considerable both in absolute terms and in relation to the weight of the organ The pulmonary vessels are very distensible and the amount of blood they accommodate must be expected to change markedly with alterations in pulmonary arterial or pulmonary venous pressures The pulmonary vasculature is therefore often looked upon as a potential blood depot It has however not been well defined how much blood may be mobilized from the lungs in various situations Nor is it known to what extent such mobilization may be influenced or regulated via vasomotor activity

Bleeding appears to be the most convenient and powerful stimulus for the mobilization of blood from vascular sections with a possible depot function Chien and Usami (1969) have used such an approach for the evaluation of alterations in central and splanchnic blood volumes Their evaluations of volume changes were however carried out about 1—2 hrs after the animals had been bled

The aim of the present experiments was to evaluate the extent of the immediate pulmonary blood volume reducti

which had been given isotope-labelled erythrocytes and albumin, were used for the experiments, and arrest of the circulation in defined situations was attempted by rapid freezing of whole animals in liquid nitrogen. The reduction of blood volume in the lungs was compared with that in the liver and in muscle tissue. A very marked decrease of pulmonary blood volume was recorded after bleeding.

Methods

Inbred male Wistar spf rats weighing 220 to 260 g were used. The animals were kept for at least 6 days in our animal house before being used in experiments. They had free access to food and water until the day of the experiment.

Anesthesia was achieved by i.p. injection of 30 mg/kg b.w. of pentobarbitone (Nembutal® Abbott, diluted 1 to 4 in isotonic saline). Such a concentration of Nembutal does not evoke pain when injected intracutaneously. The right femoral vein was exposed and cannulated, whereupon an i.v. injection of heparin (pure, powdered heparin (Novo)), 200 I.U. in 0.25 ml of isotonic saline was given.

In some initial tests the femoral arterial blood pressure was followed and recorded with a Statham P 23 pressure transducer and a Sanborn 770 Series recorder.

40 min after initiation of anesthesia a mixture of ^{51}Cr labelled rat erythrocytes and ^{125}I labelled human plasma albumin was injected i.v. with a 1 ml tuberculin syringe. Blood was withdrawn into the syringe and reinjected three times in order to obtain as complete an injection of the isotopes as possible. The syringe and the needle were then rinsed in 10 ml saline, 1 ml of which was analysed for remaining ^{51}Cr and ^{125}I.

For further procedures the rats were divided in 3 groups. Rats in the control group were not bled. In the 2 other groups standardized blood losses were arranged, a relatively moderate loss in the one group, a more marked one in the other. In the control group, a small test blood sample (0.2 ml) was withdrawn 8 min after introduction of the isotopes. Estimations of hematocrit were carried out in blood from all the rats, using microhematocrit tubes which were centrifuged for 15 min at 13 000 × g in an International Equipment Co., Model 3412 centrifuge. When this method is used, the amount of trapped plasma is so small that it can be neglected (Wintrobe 1967). Two or three blood samples were taken and their plasma and erythrocyte volumes calculated, using the weight and hematocrit values found for the samples and a density for rat blood of 1.05 (Everett, Simmons and Lasher 1956).

In the two groups sustaining hemorrhage, blood withdrawal from the femoral vein was started 5 *min after the isotope injection.* A *Harvard withdrawal pump,* Model 937, was used, and the bleeding was continued for 5 min in both groups and at constant rates calculated to give blood losses corresponding to about 12 % or 24 % of the blood volumes. Actual blood losses of 10 % and 20 % were regarded as the lowest acceptable values in the two groups, and those animals from which the bleeding had been less were discarded from the series. Such insufficient bleeding occurred occasionally either because some air was sucked into the syringe or because the animal's blood volume had been underestimated.

In the group that had lost about 12 % of the blood volume, measurements of hematocrit and isotope concentration per unit blood volume revealed no increase in plasma volume at the end of the bleeding. The shed blood was therefore regarded as representative for that remaining in the animal at this time, and the hematocrit [illegible] and erythrocytes were used directly in further c[illegible] lost about 24 % of their blood volume there [illegible] plasma volume during the 5 min bleeding pe[illegible] a blood sample of 0.2 ml was withdrawn just after the bleeding had ended, and its levels of plasma and erythrocyte radioactivity were measured. These levels were reduced by a mean of 6.6 % and 1.4 % respectively when compared to the values found in the main batches of blood *withdrawn from these animals. These reduced levels of radioactivity* were used when the tissue blood volumes for this group were calculated.

Ten min after the isotope injection the animals from all groups were placed in a horizontal position on a small perforated tray and immersed in liquid nitrogen. The bled animals were thus frozen immediately after the blood withdrawal.

The animals were kept immersed in liquid nitrogen for at least 5 min, a period which is longer than necessary for complete freezing of all tissues in a rat (Everett, *Simmons and Lasher* 1956). They were then wrapped in aluminium foil and plastic bags and kept at about −10 °C until dissected. For this dissection instruments cooled in dry ice were used. The lungs were removed and trimmed by cutting all pulmonary vessels and bronchi as close to the lung [illegible]

as possible. The lungs were then placed in separate tubes. Standardized tissue samples were taken from the muscle in the left thigh and from the liver. The heart ventricles and their contents were also dissected free from the surrounding tissue and collected in a tube for evaluation of the blood content of the heart.

Counting of radio isotopes

Total plasma volume (μl) =

$$\frac{^{125}\text{I cpm/(mg isotope-mixture)} \times \text{amount of mixture (in mg) injected}}{^{125}\text{I cpm}/\mu\text{l plasma}}$$

Plasma volume (μl) in tissue sample =

$$\frac{^{125}\text{I cpm from tissue sample}}{^{125}\text{I cpm}/\mu\text{l plasma}}$$

Erythrocyte volumes were calculated the same way from the ^{51}Cr counts in the injected mixture per μl erythrocytes and in tissue samples.

Total body hematocrit and "tissue hematocrits" were calculated as erythrocyte volume/(erythrocyte volume + plasma volume).

Results

In evaluating blood volume alterations subsequent to a blood loss it is important to know the prevailing level of the arterial blood pressure. Ideally one would want the arterial pressure to be maintained at a normal level during the test procedures. Since an anesthetic had to be used in the present experiment, it was important to know if blood pressure reductions were induced by the anesthetic. In 4 preliminary experiments therefore the arterial blood pressure was followed from about 40 min after the introduction of the anesthetic and for about 15 min. The mean arterial pressure at the end of this period ranged from 115 to 135 mm Hg. Such values are within the normal range given for rats (Zweifach 1961). It was not possible to measure the pressure in the femoral artery before anesthesia was initiated so that one could observe the effect on the blood pressure of the first dose of pentobarbitone. The devel-

* $Na_2{}^{51}CrO_4$ and ^{125}I labelled human plasma albumin were obtained from Institutt [illegible] energi, Kjeller, Norway.

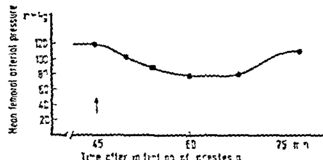


Fig. 1. The influence of *pentobarbitone on mean femoral* arterial pressure. Rat, previously anesthetized (pentobarbitone—30 mg/kg) at zero time. At arrow an additional dose of pentobarbitone *(30 mg/kg) is given.*

opment of the blood pressure on giving a similar additional dose was, however, followed in 3 animals (Fig. 1). *It can be seen that the initial blood pressure depression* had almost disappeared after 30 min. It is reasonable to assume, therefore, that arterial blood pressure was virtually unaffected by the anesthetic when 40 min after its administration, isotopes were injected and the bleeding started.

The effects of 12 % and 24 % blood losses on arterial blood pressure were then tested in another series of introductory tests. Blood withdrawal was performed in exactly the same way as in the subsequent main experiments with isotope administra*tion and nitrogen freezing of the animals. During and after 12 % blood loss the mean* arterial blood pressure did not change significantly. If anything, a slight increase in pressure was observed. When the bleeding exceeded 20 % of blood volume, however, a sudden fall in the mean arterial blood pressure of the order of 20—30 mm Hg occurred. The pressure remained at this reduced level for the following 5—10 min.

The main results of the present experiments are given in Table I and II, which present mean blood volumes and hematocrit values from the 3 groups of animals. *Some of these results need special comments. The group of animals with about 24 %* blood loss turned out by chance to have had a considerably larger mean total initial

Table I. Body weight, total blood volume, pulmonary blood volume, skeletal muscle blood content and liver blood content for the control groups and the 2 groups of bled animals. Mean values ± S.D. given for all parameters. As for "selected control group" and values in parenthesis in the second column, see text.

	Control group n = [illegible]	Group bled 11.9 % n = 14 (15)	Selected control group n = 8	Group bled 23.4 % n = 11
Body weight (g)	244 ± 11	242 ± 12 243 ± 12	238 ± 11	23[illegible] ± 13
Total blood volume (ml/100 g)	[illegible] ± 0.60	[illegible].82 ± 0.51 5.88 ± 0.5[illegible]	6.28 ± 0.46	6.27 ± 0.5[illegible]
Pulmonary blood volume (µl)	184[illegible] ± 38[illegible]	138[illegible] ± 210 1453 ± 332	2029 ± 392	128[illegible] ± 170
Muscle blood content (µl/g tissue)	2[illegible].9 ± [illegible].3	18.0 ± 5.5 18.4 ± 5.2	24.6 ± 3.2	18.9 ± 2.6
Liver blood content (µl/g tissue)	175 ± 39	139 ± 26 161 ± 27	174 ± 40	161 ± 2[illegible]

TABLE II Weight of pulmonary tissue, hematocrit values in blood from great vessels and various "tissue hematocrits" in the four groups of animals (mean values ± S D) As for "selected control group", see text

	Control group n = 17	Group bled 11 9 % n = 14	Selected control group n = 8	Group bled 23 4 % n = 11
Weight of pulmonary tissue without blood (mg)	1077±139	997±162	1095±156	886±109
Hematocrit in blood from great vessel	45 2±0 9	45 9±0 9	44 2±0 9	45 0±0 9
Pulmonary "tissue hematocrit"	31 6±5 5	35 8±3 3	28 8±4 6	36 0±2 4
Liver "tissue hematocrit"	31 4±4 5	29 7±5 7	30 8±4 4	27 1±4 8
Skeletal muscle "tissue hematocrit"	33 0±4 0	30 2±4 1	33 7±4 2	28 1±1 6

blood volume than the two other groups Such a difference must obviously reflect initial differences in the blood content of various organs and tissues In the control group of animals there was a reasonably good degree of correlation between total blood volume and pulmonary blood volume (r = +0 60 ± 0 25 (S D)) The correlation between muscle blood content and total blood volume was striking r = 0 96 ± 0 25 (S D) For our comparisons of organ blood volumes it would apparently be preferable to have groups with the same initial total blood volume A further selection within the control group was therefore carried out The 8 rats with the highest blood volumes in this group were sorted out to make a "selected control group" This "selected control group" had the same mean initial total blood volume as the group exposed to a 24 % blood loss

Individual values of pulmonary blood volume varied considerably in all groups With the exception of one single value in the group with 12 % blood loss, all these values were less than 2 S D from the mean of the group This single deviating value was more than 3 S D *from the mean* (*and more than 5 S D from the mean* when the value itself was excluded) The values for the group with a 12 % blood loss are given therefore, both with the data from this single animal included and with the same data omitted (values in parenthesis)

Fig 2 shows a comparison of mean reductions in pulmonary, muscle and liver blood volumes with the reduction in mean total blood volume The degree of plasma dilution by "autotransfusion" during the bleeding cannot be accurately calculated, and has not been taken into account here In the group with 23 4 % blood loss, therefore, the volume of blood actually remaining was somewhat higher than given in the figure

One important finding illustrated in Fig 2 is the much more marked degree of reduction in pulmonary blood volume than in total blood volume The muscle blood content is reduced to the same extent as total blood volume, whereas liver blood content is reduced far less Another remarkable thing is that the weight of the pulmonary

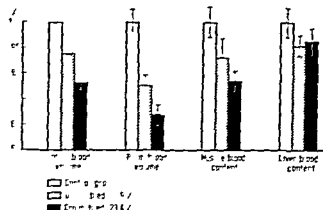


Fig. 2. Relative reductions in total blood volume, in the pulmonary blood volume and in the muscle and liver blood content upon bleeding. Bars on top represent ± S.E.

tissue is also considerably reduced in the two groups of bled animals (Table II). This can only mean that a reduction in the water content of the tissue has occurred.

Changes in the various tissue hematocrit values differ in the bled animals. The pulmonary hematocrit increased, whereas the liver and the muscle hematocrit decreased after bleeding.

An estimate of the absolute contribution from the various organs to the total blood volume reduction during the bleedings is also of interest. If the weight of the liver and of the muscle tissues were known, then the total amount of blood which is mobilized from each of the two organs could be calculated. In 3 separate control rats the weight of the whole liver was found to be approximately 4.5% of the body weight. The weight of the striated muscle can be taken as 45% of the body weight (Caster et al. 1956). Table III gives the calculations of the amounts of blood contributed by the three different tissues.

The blood content in the heart ventricles was measured in some animals from all groups. Within relatively narrow limits the values were found to be the same in all animals.

Table III. Calculated mean values for the absolute and the percentage contribution to the shed blood from the vascular beds of lungs, skeletal muscle and liver. For comparison initial blood content of the organs are also given.

	Group with 11.9% blood loss				Group with 23.4% blood loss			
	Initial blood content		Contribution to volume of shed blood		Initial blood content		Contribution to volume of shed blood	
Organ	ml	%	ml	%	ml	%	ml	%
Total body	14.1	100	1.68	100	14.8	100	3.44	100
Lungs	1.83	13	0.45	2	2.03	14	0.[illegible]4	22
Skeletal muscle	2.30	16	0.32	19	2.63	18	0.61	18
Liver	1.92	14	0.18	11	1.85	13	0.14	4

Discussion

The main aim of the present series of investigations was to get information about acute blood volume alterations in the lungs. As a first step one would like to know how readily and to what extent the pulmonary blood volume alters after an induced blood loss. Knowing the dimensions of the change, the next step would be to analyse the possible elements of active regulation involved in the pulmonary blood volume reduction.

An investigation of this type depends critically on the possibility of performing realiable and accurate measurements of blood volumes of various organs before and immediately after bleeding. The methods for blood volume estimation which can be applied repeatedly to the same animal are based on measurements of mean transit time for an injected material (Stewart 1922, Rapaport 1956). Such methods could not easily be used in the present experiment, because rapid and simultaneous measurements of blood volumes in several organs would be difficult to achieve. Also such methods have several inherent errors.

Another totally different way of approaching the problem would be to obtain a sudden arrest of the circulation at the moment when a measurement of intravascular volume was desired. Hakkila and Pietila (1961) have attempted to measure lung blood volume in this way. They suddenly stopped the circulation by i.a. injection of CaCl, removed the lungs and measured the blood content of the organ. One disadvantage with this method is that the chest must be opened, and this will inevitably cause alterations in transmural vascular pressures.

Another way of arresting circulation for subsequent blood volume measurements in various vascular beds would be rapid freezing of the total organism. This type of approach, which calls for the use of small experimental animals, has already been used for determination of the blood content in different organs by Everett *et al.* (1956). They used rats weighing from 180 to 250 g and found that the time needed for total freezing of the innermost organs was somewhat more than 2 min. One possible error of such a method could be that movement of blood from the pulmonary vascular bed to other vascular compartments occurred during freezing. Everett *et al.* tested this possibility using radio-isotope labelling of blood. They found that the radioactivity in the thoracic segment and consequently the blood volume in this part did not change during freezing.

It was consequently decided to use this method. The constancy of thoracic blood volume during freezing also indicates that pulmonary congestion does not develop during freezing. Only if the presence of pulmonary vascular congestion is ruled out can the values for pulmonary blood volume and lung tissue weight found in the present control group be regarded as normal ones. Everett *et al.* (1956) found that all heart activity, as measured by ECG, had stopped within 8 sec of immersion in liquid nitrogen. This leaves very little time for the development of a left ventricular
Also, the amounts of blood found in the ventricles in the present experimen
uniform and not particular larg

same phase of their contraction cycle and without any ventricular accumulation of blood. Furthermore, the relative value of pulmonary blood volume found, about 13 % of the total blood volume, is in good agreement with that found in other investigations, e.g. in humans (Dock *et al.* 1961, Yu *et al.* 1967) and dogs (Stewart 1922). In dogs killed after establishing acute left ventricular failure, Stewart (1922) found the pulmonary blood volume to be much larger, namely 22 % of total blood volume.

Some sort of anesthetic had to be used before freezing the animals. Chloralose or chloralose urethane is generally regarded as being very suitable in circulatory research. Their use would, however, necessitate the i.v. injection of a large volume of fluid, which should definitely be avoided in experiments of the present type. Preliminary tests showed that a small dose of pentobarbitone and a suitable waiting period gave an anesthesia in which arterial blood pressure was maintained at a normal level. This procedure was therefore adopted.

The preliminary tests also showed that a 12 % blood loss was well tolerated by the anesthetized rats, leaving their systemic arterial blood pressure unchanged. Such a blood loss was therefore chosen as the main stimulus to vascular capacity alterations in the present experiments. It is not known if pulmonary arterial and left atrial pressure altered as a result of bleeding, but these parameters have been reported to be less changed after a blood loss than systemic arterial pressure (Kallay, Takacs and Nagy 1961). Marked passive alterations in the pulmonary vascular volume resulting from reductions in transmural pulmonary pressures might therefore not be expected to occur in rats losing 12 % of their blood volume.

The results show that the blood volume in the lungs is relatively much more reduced during a bleeding of this magnitude than are the blood volumes in muscle and liver. Blood mobilized from the lungs equals 27 % of the lost blood. This is more than the amount of blood derived from the whole mass of skeletal muscle. In this situation, therefore, the lungs are certainly acting as a blood depot, their blood content being reduced by as much as 25 %. Chien and Usami (1969) found a reduction in central blood volume (right atrium-abdominal aorta) of only 9 % in dogs bled 14.5 %. Their measurements were, however, carried out about one h after the bleeding. In four animal bled 23 % they found the central blood volume reduced by 35 %, which is about equal to the pulmonary blood volume reduction (36.5 %) found in the group losing 23.4 % of total blood volume in the present investigation.

There is insufficient knowledge about the role which active regulatory mechanisms may play in the control of pulmonary vascular capacity. One would, however, expect sympathetic vasoconstrictor fibres to be involved in such mechanisms. In isolated lung preparations it has been shown (Daly and Waaler 1961, Daly, Ramsay and Waaler 1970) *that the pre-capillary and post-capillary vascular volumes can be reduced by stimulation of sympathetic nerve fibres.* In a perfused in situ lobar preparation Sndon and Fishman (1969) found that direct stimulation or reflex activation of sympathetic fibres to the lungs resulted in a less distensible and also smaller pulmonary vascular bed than prior to stimulation. Experiments on continuously weighed, isolated dog lung lobe preparations in our laboratory have shown that considera-

ble weight reductions occur on stimulation of sympathetic nerve fibres to the lungs (Nicolaysen, Waaler and Aarseth in press)

Chien and Usami found a greater reduction of the central blood volumes after a bleeding in dogs which had been sympathectomized than in dogs with their sympathetic nervous system intact These authors concluded therefore that the reduction in central blood volume after a bleeding was mainly a result of passive mechanisms However the volume of blood which is lost during a bleeding must correspond to reductions in vascular volumes somewhere It is also known that sympathetic nerve fibre activity does play a role in mobilization of blood depots from certain tissues, for example muscle (Lundgren, Lundwall and Mellander 1964 Öberg 1967) When this element of active mobilization is eliminated by bilateral removal of the sympathetic chains, then the passive reduction of vascular volumes must necessarily be greater somewhere Without knowing the normal post bleeding relationship in the various vascular beds between active vasoconstriction and passive vessel collapse, normal and sympathectomized animals cannot actually be compared in this way

One detail in the present set of results indicates that active mechanisms have played at least some role in the reduction of lung blood volume The relative mobilization of blood from the lungs was greatest during the most moderate bleeding tested, where various vascular pressures were probably best maintained According to Öberg's experiments on muscle veins (1967) passive mobilization of blood will dominate when the transmural venous pressures are low The effect of active venoconstriction became more marked at higher distension pressures Recent findings from this laboratory indicates that the same holds true for the lung vessels In an isolated perfused dog lung lobe preparation it was found that the occurrence of reductions in vascular capacity during sympathetic nerve stimulation depended on a sufficient distension pressure in the veins (Nicolaysen *et al* in press)

There is reason to believe that the sympathetic nervous system was efficiently activated in the present experiments In the rats with 23 4 % blood loss the mobilization of blood from the muscle vascular bed did thus amount to some 0 6 ml/100 g of tissue This is very similar to the vascular capacity reduction of 0 65 ml/100 g tissue (Öberg 1967) observed on stimulating sympathetic nerve fibres to an isolated cat muscle preparation with 4 imp/sec According to Lundgren Lundwall and Mellander (1964) a 20 % blood loss will cause a discharge rate of about 3—4 imp/sec in such sympathetic nerve fibres

The changes observed in the tissue hematocrits cannot be fully explained What has been measured was not the true hematocrit in the tissue blood but the ratio between the labelled erythrocytes and the sum of the labelled plasma albumin and erythrocytes Actual plasma dilution by fluid movements across the capillary membrane should thus not influence the measured 'tissue hematocrits' One possible explanation for the observed changes would be that a certain degree of erythrocyte trapping had occurred in the pulmonary vasculature after the bleedings That ...ld give fewer cells available for circulation in the remaining vascular

Several investig...

pulmonary capillaries after hemorrhagic shock (Schramel *et al* 1968, Sugg *et al* 1968)

The weight reductions of the pulmonary tissue in the bled animals deserve attention The reduction is significant at the 1 % level amounting to 19 % of lung tissue weight in the group with a 23 4 % blood loss Such a fall in weight can only be explained by a reduction of the water content in the lungs It indicates inward transcapillary flux of fluid as a result of a reduced pulmonary capillary transmural pressure The finding by Korsgren *et al* (1969) of an 88 % increase of the pulmonary extravascular fluid volume during exercise, demonstrates that this parameter may vary over a fairly wide range The weight reductions observed in the present experiments indicate that the amount of pulmonary tissue water which can be mobilized after a bleeding is also considerable This finding deserves further attention and experimental examination Not only may the lungs act as a sizable and more or less actively controlled depot for intravascular blood The extravascular fluid which th contain may apparently also vary appreciably

The work has been supported by grant from the Norwegian Research Council for Scier c a the Humanities This support is gratefully acknowledged

References

CASTER W O J PONCELET A B SIMON and W D ARMSTRONG *Tissue weights of the rat* Normal values determined by dissection and chemical methods *Proc Soc exp Biol* (N Y 1956 *91* 12?—126
CHIEN S and S USAMI Effects of hemorrhage on anesthetized dogs role of the sympathet system *Amer J Physiol* 1969 *216* 1322—1329
DALY I DE B and B A WAALER The site of action of nerves in the pulmonary vascular b of the dog *J Physiol* (Lond) 1961 *159* 46P—47P
DALY I DE B J RAMSAY and B A WAALER The site of action of nerves in the pulmona vascular bed in the dog *J Physiol* (Lond) 1970 *209* 317—339
DOCK D S W L KRAUS L B MCGUIRE J W HYLAND F W HAYNES and L DEXTE The pulmonary blood volume in man *J clin Invest* 1961 *40* 317—3?8
EVERETT N B B SIMMONS
volumes of rats determined
HAKKILA J and K PIETILA
cordial I[131] albumin dilution method compared with the blood content of the lung *logia* (Basel) 1961 *39* 125—135
KALLAY K L TAKACS and Z NAGY Pulmonary circulation in haemorrhage and haemorrhag shock *Acta physiol Acad Sci hung* 1961 *20* 155—164
KORSGREN M R LUEPKER B LIANDER and E VARNAUSKAS Pulmonary intra and extr vascular fluid volume changes with exercise *Cardiovasc Res* 1969 *3* 1—6
LUNDGREN O J LUNDWALL and S MELLANDER Range of sympathetic discharge and refle vascular adjustments in skeletal muscle during hemorrhagic hypotension *Acta physiol scand* 1964 *62* 380—390
MICHELSEN K Determin ... n the bone marrow of rabbits *Acta physiol*
NICOLAYSEN G B A WA ... ulmonary intravascular volume *A*
ÖBERG B The relationship between active constriction and passive recoil of the veins at various distending pressures *Acta physiol scand* 1967 *71* 233—247
RAPAPORT E H KUIDA F W HAYNES and L DEXTER Pulmonary red cell and plasma volumes and pulmonary hematocrit in the normal dog *Amer J Physiol* 195? *185* 1?7—132
SCHRAMEL R A HYMAN C A KELLER and W WOOLVERTON Congestive atelectasis *J Trauma* 1968 *8* 821—8?6

STEWART G N The pulmonary circulation time the quantity of blood in the lungs and the output of the heart *Amer J Physiol* 1922 *58* 20—44
SUGG W L, W R WEBB S NAKAE T THEODORIDES D N GUPTA and W A COOK, Congestive atelectasis an experimental study *Ann Surg* 1968 *168* 234—242
[illegible]
ZWEIFACH B W Aspects of comparative physiology of laboratory animals relative to the problem of experimental shock *Fed Proc* 1961 Suppl 9 18—29

Acta physiol. scand. 1970. 80. 470—491

From the Department of Pharmacology, University of Uppsala, Sweden

A Double Isotope Method for Determination of the Miscible Inorganic Sulfate Pool of the Mouse Applied to in vivo Studies of Sulfate Incorporation into Costal Cartilage

By

GEORG HERBAI

Received 27 April 1970

Abstract

HERBAI G *A double isotope method for determination of the miscible inorganic sulfate pool of the mouse applied to in vivo studies of sulfate incorporation into costal cartilage* Acta physiol scand 1970 *80* 470—491

necessary Phenol injected *i v* is converted to phenyl sulfate in the viscera and the circulating inorganic sulfate pool of the animal participates in this reaction ^{3}H phenol and ^{35}S sulfa mixed with suitable amounts of carrier are given to the animals and the tagged phenyl sulfa formed is isolated from the urine by thin layer radio-chromatography Its $^{3}H/^{35}S$ ratio togeth *with the specific activities of the injection mixture yield all necessary values for calculation* the size of the sulfate pool Simultaneously the incorporated amount of sulfate into a define portion of costal cartilage is measured after correction for the individual sulfate pool values

In 20 g NMRI mice the total miscible sulfate pools were about 300—400 μg SO_4/mous and the sulfate incorporation during the 30 min experimental period reached 200—40 nanogram SO_4 per 5 pairs of cartilaginous ribs including their osteochondral junctions Th size of the sulfate pool increased with increasing body wegiht at different ages both in NMRI and in obese hyperglycemic mice The sulfate incorporation rates showed markedly differen values in different mouse strains investigated at similar ages

Sulfation of glycosaminoglycans is a basic step during the biosynthesis of cartilage bone and different kinds of soft connective tissue ^{35}S-sulfate incorporation into carti lage has been very widely used in studies of the hormonal and metabolic control mechanisms of the sulfation process The rate of sulfate uptake into costal cartilage has been shown to be growth hormone dependent and it became a basic tool for *in vivo* growth hormone assays used by several groups (Denko and Bergenstal 1955 Murphy and Daughaday 1956 Collins and Baker 1960 Chesley 1963) Besides *in vitro* investigations have been performed on the so called sulfation factor which has been shown to reflect growth hormone activity (Salmon and Daughaday 1957, Almqvist 1961, Daughaday and Kipnis 1966

In vivo incorporation of ^{35}S sulfate into mucopolysaccharides was studied after administration of antirheumatic drugs: cortisone and hydrocortisone (Schiller and Dorfman 1957, Boström *et al.* 1964), prednisone and methylprednisolone (Wirz *et al.* 1962, Waitzman and Jackson 1967), salicylate, phenylbutazone and oxyphenylbutazone (Wirz *et al.* 1962, Boström *et al.* 1964), chloroquine and mepacrine (Whitehouse and Boström 1965).

The influence of the following hormones on *in vivo* incorporation of ^{35}S into connective tissue was studied: thyroxine (and thiouracil) (Dziewiatkowski 1951), thyrotropin (Singh and McKenzie 1969), estradiol (Priest and Koplitz 1960, Berntsen 1968) and androgens and anabolic steroids (Salmon *et al.* 1963, Wagner *et al.* 1968). The *in vivo* sulfate incorporation technique was also utilized for clarifying the effects of scurvy (Friberg and Ringertz 1954), vitamin A (McElligott 1962) and vitamin K (Kovacs *et al.* 1967) on mucopolysaccharide synthesis.

In vivo sulfate incorporation was furthermore studied in alloxan diabetes (Schiller and Dorfman 1955) and lathyrism (Berntsen 1967), after gold thioglucose administration (Wagner *et al.* 1969), following histamine injections (Khokhar and Hilker 1967) and after thalidomide feeding (Brode 1969).

The common characteristics of all these studies are that they were performed using ^{35}S sulfate without any regard to the endogenous sulfate pool of the animals. Thus the specific activity of the sulfate which reached the studied tissue was unknown.

Many of the above mentioned compounds influence renal excretory mechanisms (Takeda 1964), skeletal mineral kinetics (Gordan and Eisenberg 1963) and water and electrolyte contents of the plasma (Woolley and Timiras 1964). Furthermore it has been established that many steroid and also non-steroid hormones become conjugated to sulfate esters in the body (Boström and Westermark 1961, Boström 1965). This reaction consumes a part of the available sulfate store of the animals and thus might influence the incorporation of ^{35}S sulfate into cartilage after large doses of hormones. These facts make it necessary to measure and control the sulfate pool of the animals in all sulfate incorporation experiments. Since suitable chemical methods for sulfate determination in small plasma samples are lacking, a new double isotope method for determination of the inorganic sulfate pool of small animals is presented and evaluated in this paper. In the second part the method is used for studies on pools and incorporation rates under varying conditions. Finally the size of sulfate pools and incorporation rates in some different mouse strains are presented.

Theoretical basis and principle of the method

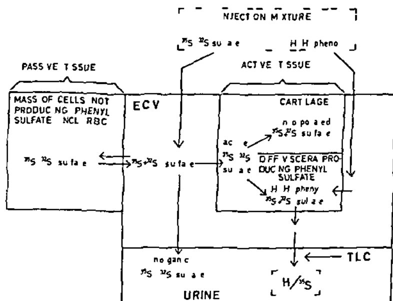


Fig 1 Schemat c model of d fferent sulfate and some phenol compartments in the mou e n d cat ng tl e princ ple of the method The dotted l nes emb ace those quant tat e data wh ch are used for calculat on of the sulfate pool RBC = red blood corpuscles ECV = extracellular volume TLC = thin layer chromatography

creted into the ur ne by f ltrat on and tubular secret on (Sperber 1948) The spec f c a t v ty c the sulfa e reco ered as phenyl sulfate w ll be found to be altered as compared to tl e spec f act ty of the injected dose by the part c pat on n the detoxicat on react on of sulfa e f or ... ne b ound

DP\ on o wh ch

thus conta ns $\frac{F \times T}{f}$ moles of phenyl rad cals and the same amount of sulfate rad als Like v se t conta ns $\frac{Z \times S}{s}$ moles exogenous sulfate ons The rat o n the phenyl sulfa e spot be tween the exogenous and total sulfate s equal to that in the wl ole an mal

$$\frac{\text{exogenous SO n the spot}}{\text{total } SO_4 \text{ n the spot}} = \frac{\text{exogenous SO n the wl ole body}}{\text{exogenous } SO_4 + SO_4 \text{ pool}}$$

Us ng the abo e chosen symbols the follow ng formula can be obta ned and then sol ed fo the total sulfate pool P_s

$$\frac{\frac{Z \times S}{s}}{\frac{F \times T}{f}} = \frac{S}{S \quad P_s}$$

$$P_s = F \times \frac{T}{Z} \quad \frac{s}{f} \quad S \tag{1}$$

P_s is obtained in moles When the urine sample for pool determ na on l as been l d awn further ncorporat on of circulating ^{35}S sulfa e into cart lage is nterrup ed by nject on of a large amount of unlabeled sod um sulfate wh ch mmed ately decreases the spec f c a t of

the circulating ^{35}S sulfate After killing the animal a defined amount of costal cartilage is dissected out and its content of ^{35}S measured Now the amount of sulfate which has been incorporated into the sample during the experimental period can be calculated using the following equation

$$U = 1000 \times g \times \frac{S + P_s}{s} \quad (2)$$

where U = incorporated sulfate ions of cartilage in nanomoles g = DPM ^{35}S in the cartilage samples S = injected sulfate in μmoles s = injected ^{35}S in DPM and P_s — sulfate pool of the animal in μmoles

Materials and methods

Animals

Their age varied from weaning

In some experiments obese

and stored in a room

rhythm was maintained

Diet

free access to tap water

Injections

All *i v* injections were performed by the orbital plexus technique (Pinkerton and Webber 1964) using a 0 3 mm Ø injection needle The quenching dose of sodium sulfate was administered *i p* The steroid hormones were dissolved in olive oil and injected *s c*

Blood and urine samples

constric

7 mm)

the mouse

out diffi

culty

Isotopes

Carrier free ^{35}S sulfate and ^{3}H labeled phenol (Ring T(G) spec act 250 mC/mmole) were obtained from the Radiochemical Centre Amersham England ^{3}H phenol was dissolved in

r to minimize radiolysis The

using the following solvent

of phenol 0 60 Radiolysis

($^{35}SO_4$) and of ^{3}H (n hexa

Amersham for calibration of

the spectrometer in order to adjust the counting results to 100 % efficiency (DPM)

Drugs and chemicals

Testosterone propionate estradiol benzoate and hydrocortisone acetate were obtained from Organon Co Oss Holland

Na_2SO_4 10 H_2O and phenol barium acetate and 2 naphthylamine were purchased from Merck A G Germany Aniline-HCl and p-hydroxybenzoic acid were from AB Kebo Stockholm DL tyrosine was from Sigma Chem Co, USA and phenyl sulfate was

Mann Res Lab USA All except tyrosine were of analytical grade

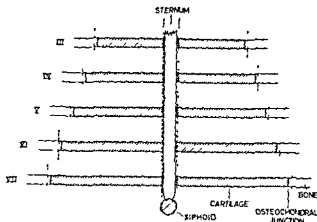


Fig 2 Schematic picture of the studied parts of the cartilaginous rib cage of the mouse The dotted lines indicate that portion of costal cartilage which was included as standard sample in the routine procedure

Thin layer radiochromatography (TLC)

TLC was performed on A G, Germany)
using plates (20 × 5 cm) in an exsiccator
and activated at 110° C ys the ascending
technique over a distance lter paper The
standard solvent system 10 10 · 5 2
(v/v) (Wusteman *et al* 1964) The aniline conjugate was separated by the following system n butanol—propan 1-ol—0 1 N NH3, 2 1 1 (v/v) (Boyland *et al* 1957) Urine samples were
vith 30 μl methanol which con
ate the trapping of the radio-
was transferred to the starting
is simultaneously run on a dif
ferent lane and the spot visualized by UV-lamp After development various suitable zones, depending on the experiment were scraped from the plates directly into liquid scintillation vials The zonal borders for the standard method are seen in Fig 4 After adding 5 ml of a liquid scintillation solution (Herbai 1970b) which causes elution of phenyl sulfate the radioactivity of the samples was counted as described below

Quenching in vivo by non radioactive sodium sulfate injection

After taking the urine sample for determination of the sulfate pool all animals received an *i p* injection of 80 mg Na_2SO_4 10 H_2O in 1 ml water in order to rapidly decrease the specific activity of the circulating $^{35}SO_4$ in the animal

Cartilage preparation

ed free *in situ* and the caudal 5
il junction and a small piece
solution the ribs were cleaned
xture as described previously
(Herbai 1970b In some experiments cartilage prepared from different organs was studied The tracheas were prepared free from the epiglottis to the bifurcation and xiphoids were cut at the bony border of the sternum When ear cartilage was studied both pinnae were cut out
the sample was cleaned
o constant
as about 2
hole blood

Radioactivity measurements

flasks when the ^{35}S sulfate content of different
or measuring the $^{3}H/^{35}S$ content of the
4 hrs in the refrigerator part of a liquid
USA) and counted until at least 20,000

[illegible] one channel Counting efficiency for ^{35}S was about 60 % when a [illegible]

All counting results were referred to 100 % absolute efficiency (DPM) This permitted the calculations of the results in chemical terms (as μg and ng)

Routine procedure for calculation of sulfate pool and sulfate incorporation

Female N M R I mice weighing about 20 g received an *i v* injection (consistently at 9 00 *a m*) which contained 20 μC ^{35}S with 96 μg (1 μmole SO_4 and 20 μC ^{3}H phenol with 376 μg (4 μmole) phenol in 0 1 ml 0 9 % NaCl (2 μl aliquots were taken during the preparation of the injection mixture for measuring its exact content of ^{3}H and ^{35}S) Thirty min later a [illegible]

carried out by a computer program

Results

Choice of sulfate trapping agent

Many different compounds are able to form sulfate conjugates in animals (see Williams 1959 and Bostrom and Westermark 1961 for ref) Before deciding on the use of phenol in the method the following substances were chosen for closer investigation p hydroxybenzoic acid, tyrosine, 2 naphtylamine aniline and phenol The compounds were injected to mice in different amounts simultaneously with ^{35}S sulfate The urines were investigated by TLC using different solvent systems After *i v* injections of non toxic doses only aniline and phenol yielded sulfate conjugates in amounts which seemed to be sufficient for quantitative studies Comparative experiments were then carried out with aniline and phenol regarding their sulfate trapping ability in the following way

Adult mice were divided in 3 groups and injected *i p* with aniline (7 7 μmoles) or phenol (8 0 μmoles) both per 10 g b w Controls received only saline Simultaneously 10 μC ^{35}S/1 μg carrier SO_4/0 1 ml H_2O was given *i v* per 10 g b w Urine samples were then drawn at different time intervals From pooled urine of each group a 4 μl sample was subjected to TLC The same amount was also counted directly Zonal analysis of the chromatograms detected distinct ^{35}S conjugates of both compounds Fig 3 *shows that phenol trapped more than 4 times more sulfate than did a nearly* equimolar amount of aniline At the peak of its secretion phenyl sulfate contained 73 % of all excreted sulfate in the urine Because of the high sulfate trapping ability phenol seemed to be the most suitable conjugate yielding agent for the method

TLC of phenyl sulfate

After *i v* injection of the standard mixture of ^{3}H phenol and ^{35}S sulfate to a mouse a urine sample was taken at 30 min and chromatographed Thereafter 4 mm frac-

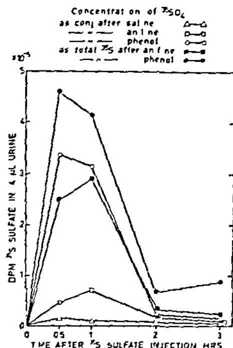


Fig 3 The effect of aniline and phenol administration on the urinary excretion pattern of ^{35}S sulfate after i.c ^{35}S-sulfate injection in the mouse Aniline dose 7 7 µmole/10 g b w Phenol dose 8 0 µmole/10 g b w Both given i p simultaneously with the i c injection of ^{35}S-sulfate

tions were scraped along the whole plate into separate scintillation vials and then counted Fig 4 demonstrates the results of a detailed zonal analysis of a TLC plate It is seen that ^{3}H phenol occurs in the form of two distinct conjugates one is associated with sulfate (R_f value 0 48) and the other probably is glucuronide (R_f= 0 18) The chemical identity of the latter has not been analyzed No free phenol or additional disturbing conjugates are found anywhere on the plate Since the shape of the phenyl sulfate spot is distinct without tailing 3 fractions (indicated by roman numerals in the fig 4 are scarped and counted in the standard procedure The fraction containing the peak was consistently used in the equation

Background activity in the phenyl sulfate spot following TLC

It has been previously shown that different mammals normally excrete small amounts of endogenous phenyl sulfate in the urine the phenol is probably produced by bacteria in the gut (Bostrom *et al* 1963) A certain amount of endogenous unlabeled phenol is thus competing with the injected ^{3}H phenol for ^{35}S-sulfate and it is found in the spot. This background" may influence the ^{3}H/^{35}S ratio Furthermore certain hormones, which form sulfate conjugates when injected in large amounts might also interfere

Injections of merely ^{35}S sulfate (without any phenol) were given to various groups of mice to check whether different conditions (see Table 1) would influence the background activity of the phenyl sulfate spot In the absence of exogenous phenol

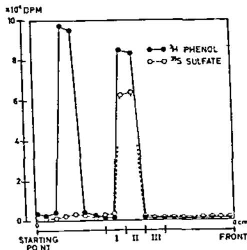


Fig 4

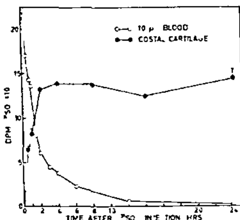


Fig 5

Fig 4 The pattern of ^{3}H phenol and ^{35}S sulfate activity on a TLC plate after development of 10 μl urine taken at 30 min The mouse had received a standard dose of the isotope mixture Fractions I II and III of each urine sample are scraped during the standard procedure and counted.

Fig 5 Disappearance of ^{35}S-activity from mouse blood after *i v* injection of ^{35}S-sulfate and the time course of the ^{35}S sulfate uptake into costal cartilage of mice killed at different intervals 4 mice per group Vertical bars indicate S E M

a certain ^{35}S activity was still found in the phenyl sulfate spot The nature of this activity was now investigated *in vitro* by adding carrier sodium sulfate or barium acetate to the urine samples prior to the TLC procedure Furthermore the effect on the chromatogram of *in vitro* added ^{35}S was checked as follows

Nine groups of female mice around 25 g (3 or 4 mice per group) were subjected to different conditions indicated in Table I As control served a group which received *i v* 4 μmoles of phenol All animals received *i v* 10 μC ^{35}S-sulfate with 10 μg SO_4/10 g b w and 30 min urine samples were pooled in each group 10 μl urine samples were then counted directly or subjected to the standard TLC procedure with subsequent zonal analysis of the spot corresponding to phenyl sulfate

Table I shows that neither starvation nor the special diet containing low amounts of phenolic materials resulted in less ^{35}S in the spot than the standard pellet diet The pre-treatment with large amounts of steroid hormones also had negligible effect

A large dose of sodium sulfate added *in vitro* to the urine did not decrease the ^{35}S background in the spot neither did a large dose of barium acetate which was supposed to precipitate inorganic ^{35}S sulfate Finally all added inorganic $^{35}SO_4$ activity remained at the starting point after development of the TLC These results suggest that the small amount of ^{35}S activity consistently found on the phenyl sulfate spot mainly consists of endogenously synthetized phenyl sulfate The smallness of this back

TABLE 1 The influence of starvation, diet composition and administration of various steroid hormones on the ^{35}S-sulfate content of the phenyl sulfate spot in the absence of carrier phenol in the injection mixture and the effect of *in vitro* addition of carrier Na_2SO_4 or barium acetate to the urine on the ^{35}S activity of the spot Q = DPM in the spot without exogenous phenol/DPM *following in vivo exogenous phenol*, %

		Commercial pellet diet	Special diet for one week	Starvation for 48 hrs	376 µg (4 µmole) phenol *i v*	8 × 10⁶ DPM $^{35}SO_4$*	1 mg Na_2SO_4/0 1 ml*	1 mg barium acetate/0 1 ml*	1 mg estradiol benzoate**	200 µg hydrocortisone acetate**	1 mg testosterone propionate**
10 µl urine	Counted directly total ^{35}S DPM × × 10^{-4}	3 26	3 41	4 03	3 41	3 00	3 67	3 88	33 9	2 83	3 67
	Developed by TLC ^{35}S in the spot DPM	*410*	*593*	*663*	*2 99 × × 10⁴*	*0*	*493*	*465*	*615*	*580*	*445*
Spot/total %		1 26	1 74	1 64	87 68	0	1 34	1 20	1 81	2 01	1 21
Q		1 37	1 98	2 22	—	0	1 65	1 55	2 06	1 94	1 49

* added to the urine *in vitro*
** per animal and day for 6 days prior to the experiment

ground activity in contrast to the high phenyl ^{35}S-sulfate quantity in the spot after a large carrier phenol injection makes its influence upon the sulfate pool calculations negligible

Sulfate and phenyl sulfate kinetics in the mouse

Since it has been reported that the major portion of *i v* administered ^{35}S sulfate rapidly disappears from the blood of the guinea pig (Zumel 1965) the following experiments were performed in order to establish the optimum experimental circumstances for the standard method in the mouse

23 female mice weighing about 24 g were injected *i v* with 10 µC ^{35}S with 0 01 µg SO_4 per 10 g b w The animals were divided into 7 groups 5 µl blood samples were drawn from the mice at different time intervals (5 min—24 hrs) and pooled from each group A 10 µl aliquot from each pooled blood sample was then oxidized and analyzed for ^{35}S content The mice were killed at different intervals without sulfate quenching (see below and the ^{35}S content of the usual costal cartilage samples was determined The results are summarized in Fig 5 which shows a rapid decline of the ^{35}S content of the blood during the first 2 hrs The incorporation of ^{35}S-sulfate into the costal cartilage is also rapid during the first 2 hrs and then reaches a plateau presumably because of a rapid disappearance of the circulating ^{35}S-sulfate The labelling of costal cartilage is thus practically terminated at 2 hrs

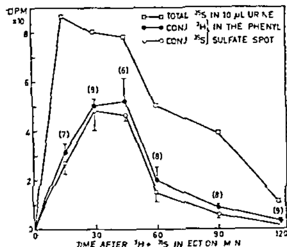


Fig 6 Time course of the excretion of ^{3}H phenyl ^{35}S-sulfate and total $^{35}SO_4$ in the urine after i.v. injection of ^{3}H phenol and ^{35}S sulfate Total $^{35}SO_4$ was measured in pooled urine samples The figures in brackets indicate the number of TLC separated samples at each time and the vertical bars indicate S E M

The time course of the phenyl sulfate excretion into the urine was studied in 10 female mice weighing about 24 g They received i.v. 10 µC ^{3}H phenol with 1 µmole carrier and then separate urine samples were taken at different intervals One drop of urine from each animal was pooled at all times for direct counting of the total ^{35}S content of the urine The individual samples were separately developed on TLC plates and the ^{3}H and ^{35}S contents of the phenyl sulfate spots were measured The results are seen in Fig 6 It shows a rapid and parallel increase of the ^{3}H phenol and ^{35}S sulfate content of the spot in the beginning reaching a maximum at 30—45 min and then a sharp decline At 60 min the major part of the conjugate has been excreted In another experiment the standard injection mixture was given to a group of female mice weighing about 22 g Urine samples were then taken at different intervals and TLC separated The influence of the time for taking the urine sample upon the $^{3}H/^{35}S$ ratio of the spot was now investigated It can be seen from Table II

TABLE II The influence of the time between the injection of the standard mixture and the taking of the urine sample on the $^{3}H/^{35}S$ ratio in the phenyl sulfate spot and on the calculated size of the sulfate pool

	Time between the ^{35}S sulfate injection and taking the urine sample min					
	15	30	45	60	90	120
number of urine samples	4	4	6	8	9	9
$^{3}H/^{35}S$ ratio in the spot mean ± S E M	1 35±0 07	1 37±0 08	1 33±0 12	1 33±0 12	1 10±0 12	0 93±0 10
Mean sulfate pool µg ± S E M	282±20	287±23	268±25	251±24	209±27	164±18

TABLE III The effect of 80 mg $Na_2SO_4 \cdot 10H_2O$ per mouse administrated i p at different time intervals after the $^{35}SO_4$ injection on the level of incorporated ^{35}S sulfate into costal cartilage samples The sodium sulfate dose corresponds to 24 mg SO_4 ions

group	Time for quenching after $^{35}SO_4$ injection	Number of animals	Body weight g mean ± S D	DPM $^{35}SO_4$ per cartilage sample mean ± S E M
A	30 min	11	18 0 ± 2 3	8 700 ± 640
B	no quenching	6	16 2 ± 2 3	19,700 ± 1 690
C	8 hrs	10	16 8 ± 1 4	19 400 ± 870

that the ratios and the calculated sulfate pool values were reasonably constant during the first hour but then decreased This experiment indicates that a standard time of 30 min for taking the urine sample is convenient for calculations and that small time deviations are not critical The fact that already at 15 min a constant pool value is obtained indicates that the injected $^{35}SO_4$ equilibrates with the $^{32}SO_4$ pool of the animal before any appreciable amount of ^3H phenyl sulfate is formed

Quenching of the circulating $^{35}SO_4$ by Na_2SO_4 injection

The reason for this procedure is the following The sulfate pools used to be calculated from urine samples taken at 30 min but for reasons to be discussed, the animals were left alive until about 4 hrs after the isotope injection It was therefore necessary to stop further $^{35}SO_4$ incorporation into the cartilage after 30 min in order to limit the sulfate incorporation to a period of time close to that where the pool determination is done The following experiment is concerned with the quenching procedure Three groups of male mice received 10 μC ^{35}S with 0 1 μmole SO_4 both per 10 g b w One group (B) was killed without quenching another (A) received a quenching dose of sulfate at 30 min and the third group (C) obtained the same quenching dose after 8 hrs All animals were killed at 24 hrs The results are shown in Table III which demonstrates that quenching at 30 min terminates the isotope uptake into the cartilage at a level of 44 % of that without quenching If however the quenching dose was given 8 hrs after the $^{35}SO_4$ injection when the incorporation of ^{35}S-sulfate had come to an end the large dose of inorganic sulfate apparently did not "push" out the previously incorporated sulfate from the tissue

Incorporated sulfate levels in different types of cartilage

Cartilage tissue from different anatomical sources has been used in previous studies In different types of cartilage (e g actively growing or "static") different turnover rates of sulfate might be anticipated It seemed therefore to be of interest to study ^{35}S-sulfate incorporation into cartilage specimens dissected from different organs 35 growing female mice weighing about 20 g received an injection of the standard mixture and 30 min later an additional carrier free dose of 50 μC ^{35}S sulfate 30 min later the usual quenching sulfate dose was administered and the mice were divided into 7 groups which were then killed at different time intervals ranging between 1

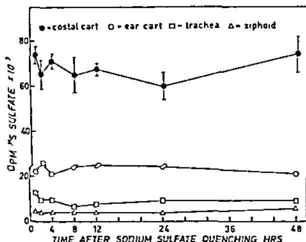


Fig 7 The levels of incorporated ^{35}S-sulfate following the SO_4 quenching in cartilage specimens prepared from different organs Each point represents the mean values of 5 pooled tracheas, xiphoids or ear cartilage samples taken from 5 mice The sulfate uptake of ear samples is expressed as DPM ^{35}S/10 mg dry tissue The costal samples were processed and counted individually and the vertical bars indicate S E M

and 48 hrs Ear cartilage, xiphoids, tracheas and standard costal cartilage samples were prepared, as described above, for determination of the ^{35}S content Fig 7 shows constant sulfate levels during the first 48 hrs in all types of cartilage which indicates that no initial differences in turnover rates of incorporated sulfate occurred between the cartilage samples of different origin Costal cartilage incorporated the highest amounts of sulfate probably because of its plentiness, hence it serves as the most convenient source of cartilage tissue for radio-sulfate studies

Influence of different amounts of carrier phenol and sulfate

The following experiments were devoted to determination of the optimum concentration of the labeled and carrier compounds in the injection mixture Since phenol is a toxic agent it was necessary to evaluate how increasing amounts of it influence the formation of conjugates their excretion pattern into the urine and the calculated values of the sulfate pool In addition, the effects of phenol on the sulfation activity of costal cartilage were studied Seven groups of growing male mice received i v a mixture of $^{35}SO_4$ and ^{3}H phenol (Composition see text of Table IV) Simultaneously the different groups received *increasing amounts of phenol injected into the other* orbital plexus Urine samples were drawn at 30 min and the pooled samples of each group were subjected to the usual TLC procedure and subsequent zonal analysis of the entire plates These experiments are summarized in col 2—17 of Table IV Additional groups of similar mice were treated as quoted above for investigating the influence of phenol upon the sulfate uptake of cartilage The results of the latter experiment are summarized in col 18—20 of Table IV In the first part of the table (columns 3—9) the values concerned with the ^{3}H phenol (conjugated and unconjugated) of the urine are given It is seen in col 4 that increasing amounts of injected phenol elicit a gradual elevation in the phenol content of the urine Practically all phenol is excreted as glucuronide' and sulfate conjugate, in about equal proj

TABLE III The effect of 80 mg Na_2SO_4 $10H_2O$ per mouse administrated i p at different time intervals after the $^{35}SO_4$ injection on the level of incorporated ^{35}S sulfate into costal cartilage samples The sodium sulfate dose corresponds to 24 mg SO_4 ions

group	Time for quenching after $^{35}SO_4$ injection	Number of animals	Body weight g mean±S D	DPM $^{35}SO_4$ per cartilage sample mean±S E M
A	30 min	11	18 0±2 3	8 700±640
B	no quenching	6	16 2±2 3	19 700±1 690
C	8 hrs	10	16 8±1 4	19 400±870

that the ratios and the calculated sulfate pool values were reasonably constant during the first hour but then decreased This experiment indicates that a standard time of 30 min for taking the urine sample is convenient for calculations and that small time deviations are not critical The fact that already at 15 min a constant pool value is obtained indicates that the injected $^{35}SO_4$ equilibrates with the $^{32}SO_4$ pool of the animal before any appreciable amount of ^{3}H phenyl sulfate is formed

Quenching of the circulating $^{35}SO_4$ by Na_2SO_4 injection

The reason for this procedure is the following The sulfate pools used to be calculated from urine samples taken at 30 min but for reasons to be discussed the animals were left alive until about 4 hrs after the isotope injection It was therefore necessary to stop further $^{35}SO_4$ incorporation into the cartilage after 30 min in order to limit the sulfate incorporation to a period of time close to that where the pool determination is done The following experiment is concerned with the quenching procedure Three groups of male mice received 10 μC ^{35}S with 0 1 μmole SO_4 both per 10 g b w One group (B) was killed without quenching another (A) received a quenching dose of sulfate at 30 min and the third group (C) obtained the same quenching dose after 8 hrs All animals were killed at 24 hrs The results are shown in Table III which demonstrates that quenching at 30 min terminates the isotope uptake into the cartilage at a level of 44 % of that without quenching If however the quenching dose was given 8 hrs after the $^{35}SO_4$ injection when the incorporation of ^{35}S sulfate had come to an end the large dose of inorganic sulfate apparently did not push out the previously incorporated sulfate from the tissue

Incorporated sulfate levels in different types of cartilage

Cartilage tissue from different anatomical sources has been used in previous studies In different types of cartilage (e g actively growing or static) different turnover rates of sulfate might be anticipated It seemed therefore to be of interest to study ^{35}S sulfate incorporation into cartilage specimens dissected from different organs 35 growing female mice weighing about 20 g received an injection of the standard mixture and 30 min later an additional carrier free dose of 50 μC ^{35}S sulfate 30 min later the usual quenching sulfate dose was administered and the mice were divided into 7 groups which were then killed at different time intervals ranging between 1

estimated causing an apparent increase in specific activity This part of the experiment shows that the injected dose of phenol must not be too small Similar results are obtained if the $^3H/^{35}S$ ratio of the spot is used in the usual procedure for calculation of the sulfate pool Col 17 demonstrates that the calculations yield incorrect pool values if the exogenous phenol dose is below *188 µg/10 g b w* (rows B and C)

The fourth part of Table IV (col 18—20) shows that increasing amounts of carrier phenol up to 376 µg do not influence the ^{35}S uptake into the cartilage samples If the chemical amount of sulfate incorporated into the cartilage was calculated using the mean sulfate pool values, it is seen in col 20 that only the two highest phenol doses caused a slight, probably insignificant, decrease of sulfate incorporation into cartilage At these doses however, the animals showed tremor and their urine had a brownish colour Rows B and C of col 20 are left empty because the pool values were incorrect

The influence of *the sulfate concentration in the injection mixture* was investigated in the following way Four groups of female mice were injected with increasing amounts of sulfate and phenol in constant proportions After the usual procedure the sulfate pools and the sulfate levels of cartilage were calculated The results which

^{35}S sulfate content of 10 µl urine		*Sulfate distribution on thin layer plate after chromotagraphy of 10 µl urine*				*$^3H/^{35}S$ ratio of the penyl sulfate spot*	*Calculated sulfate pool µg/10 g b w mean value*	*Sulfate incorporation into costal cartilage*		
Total ^{35}S DPM × 10^{-4}	conj / total ^{35}S %	Total ^{35}S on the plate DPM × 10^{-4}	^{35}S as phenyl sulfate DPM × 10^{-4}	conj / total ^{35}S %	Specific activity of SO_4 in *the spot* DPM/mg			Number of animals	^{35}S per sample DPM mean ± S E M	Calculated sulfate uptake ng
10	11	12	13	14	15	16	17	18	19	20
1 45	0 3	2 45	0 05	1 9	—	—	—	11	2 050 ±150	—
3 00	0 5	2 00	0 14	7 0	59 58	97 1	45	4	1 930 ±133	—
2 50	8 6	3 07	2 15	69 9	9 15	6 4	353	4	2 220 ±117	—
2 17	8 6	2 50	1 87	74 8	7 07	4 3	472	6	2 220 ±160	321
1 80	41 5	8 52	7 47	87 7	8 12	1 9	408	13	2 380 ±117	298
1 75	29 5	5 85	5 17	88 4	6 44	1 1	475	11	1 980 ±183	288
1 60	60 6	10 80	9 70	89 8	7 24	0 5	459	10	1 970 ±123	278

TABLE V The effect of different concentrations of the injected sulfate phenol mixture on the $^3H/^{35}S$ ratio of the urinary phenyl sulfate on the calculated size of the sulfate pool and on the estimated value of sulfate incorporated into costal cartilage

Group	Number of mice	Body weight g ± S E M	Composition of the mixture injected i p (0 1 ml/mouse)				$^3H/^{35}S$ ratio in the spot ± S E M	Sulfate pool μg ± S E M	Incorporated sulfate per cartilage sample ng ± S E M
			DPM ^{35}S	DPM ^3H	μg sulfate	μg phenol			
A	7	20 7 ± 0 2	1 1 $\times 10^7$	8 6 $\times 10^6$	24	94	4 10 ± 0 3	459 ± 39	245 ± 25
B	6	21 7 ± 0 3	2 1 $\times 10^7$	1 7 $\times 10^7$	48	188	1 91 ± 0 1	403 ± 25	238 ± 48
C	6	20 2 ± 0 4	4 3 $\times 10^7$	3 4 $\times 10^7$	96	376	1 17 ± 0 05	456 ± 24	216 ± 20
D	6	21 0 ± 0 8	8 6 $\times 10^7$	6 8 $\times 10^7$	192	752	0 72 ± 0 03	490 ± 28	229 ± 18

are summarized in Table V show that increasing concentration of the components yielded decreasing $^3H/^{35}S$ ratio in the spot, but neither the sulfate pool values nor the cartilage sulfate levels were affected This is a great advantage and permits the injection of a fixed volume of the mixture without respect to the body weight variations of the experimental animals As standard mixture in mouse experiments the composition of group C has been chosen, since it yielded convenient amounts of radioactivity in the cartilage and in the phenyl sulfate spot with optimum separability of ^3H and ^{35}S in the spectrometer

Loading the animals with large amounts of sulfate

Despite the rapid elimination of injected sulfate in the mouse (see Fig 5) an attempt was made to measure the appearance of exogenous sulfate in the pool after administration of large amounts of inorganic sulfate at different times in relation to the isotope experiment The results are seen in Table VI 300 μg SO_4 injected 5 min prior

TABLE VI The appearance in the sulfate pool of extra amounts of injected inorganic sulfate given at different intervals before the injection belonging to the standard procedure The table also shows the influence on the calculated incorporation values of sulfate into costal cartilage

In vivo extra sulfate load i t	Number of animals	Mean body weight g ± S E M	Mean sulfate pool μg ± S E M	Mean of calculated sulfate uptake per cartilage sample ng ± S E M	Increase in pool size μg	Increase in pool size as % of the injected dose
Controls 0 1 ml saline	12	22 5 ± 0 3	193 ± 8 5	107 8	—	
300 μg SO_4 5 min prior to isotope injection	6	22 0 ± 0 5	428 19	121 15	235	78 3
1 200 μg SO_4 5 min prior to isotope injection	6	21 9 0 5	908 47	199 21	715	59 6
1 200 μg SO_4 30 min prior to isotope injection	7	20 9 ± 0 5	479 ± 18	102 8	286	23 8

to the isotope mixture caused a considerable elevation of the sulfate pool The estimated sulfate incorporation was somewhat elevated but the difference is not statistically significant 78 % of the injected extra sulfate was found in the sulfate pool 1200 μg SO_4 injected 5 min before the mixture elicited a high elevation of the sulfate pool and a significant increase in the calculated uptake of sulfate About 60 % of the injected dose was found in the sulfate pool If the increase in sulfate uptake is real and not an artefact, it could depend on the transient very high sulfate content in all tissues including the costal cartilage But since the pool was determined under far from steady state conditions the results may be spurious It will have to be checked by a more direct method When the same large amount of sulfate was injected 30 min prior to the isotopes, a considerable part of it had already disappeared from the body, and no influence upon the incorporation could be recorded In this case only 24 % of the injected extra sulfate was found in the pool

Correlation between body weight and sulfate pool

The method was utilized for determinations of the sulfate pool sizes in animals of different body weights and ages N M R I mice of both sexes were used in this study which was performed at different occasions during a period of 2 months The weight of the mice ranged between 8 0 and 32 g The results from 54 male and 58 female mice of different ages are presented in Fig 8a and b In Fig 8a the values are plotted on a double logarithmic scale Increasing body weights are associated with elevation of the sulfate pools in both sexes The relative scatter is of the same order of magnitude at different weight levels In Fig 8b the pool size/g body weight is shown It shows a very slight elevation with increasing body weights in both sexes

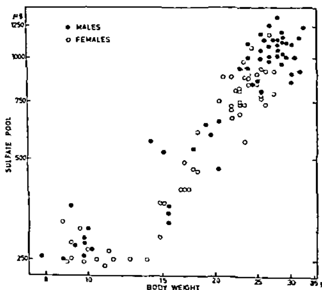


Fig 8a Correlation between the body weight and size of the total sulfate pool in male and female N M R I mice of different ages (3—12 weeks) plotted on a double logarithmic scale

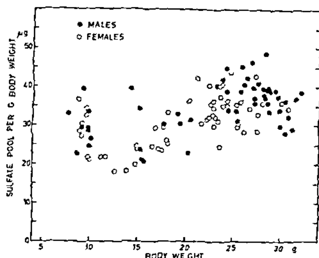


Fig 8b Correlation between the body weight and the sulfate pool per g body weight in the same material as presented in Fig 8a plotted on a linear scale

Obese hyperglycemic mice are characterized by extreme fat deposition with a consecutive increase of body weight The syndrome becomes manifest during the most intense growth activity of young animals It seemed to be of interest to investigate the sizes of the sulfate pools in the obese mice and their lean litter mates during a major part of their life span The results are shown in Fig 9 and plotted on a double logarithmic scale A clearcut correlation can be seen between log body weights and log pools in both the obese and the lean mice It is evident that all the points fall

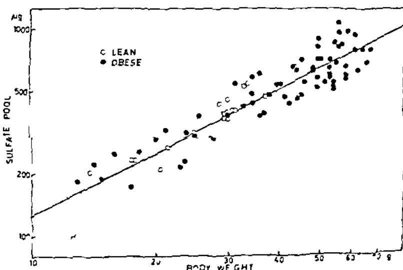


Fig 9 Correlation between the body weight and size of the total sulfate pool in obese hyperglycemic mice and their lean litter mates plotted on a double logarithmic scale The lean and obese data together were used for calculation of a common regression line

TABLE VII Sulfate uptake into costal cartilage and sizes of the sulfate pool in growing and adult N M R I mice of both sexes and in two different strains of mice *ob* = normal litter mates to obese-hyperglycemic mice *du*/Jackson = normal litter mates to dwarf mice

Mouse strain	Age and sex	Number of experiments	Body weight g S D S E M	Sulfate pool µg S D S E M	Sulfate pool per g b w S D S E M	Sulfate uptake per cartilage sample ng S D S E M
	4 weeks		23 2	412	18 0	265
N M R I	growing	116	2 7	161	7 2	83
	males		0 3	15	0 7	7 9
	4 weeks		20 7	345	16 7	222
N M R I	growing	116	1 6	149	7 4	60
	females		0 1	14	0 7	5 6
	8 weeks		32 5	420	13 1	171
N M R I	adult	20	2 5	167	5 7	39
	males		0 6	37	1 3	8 7
	8 weeks		27 5	493	18 0	144
N M R I	adult	25	1 7	72	2 6	21
	females		0 3	14	0 5	4 1
ob/ lean	8 weeks		19 5	327	17 3	79
litter mates	adult	22	2 2	96	4 1	20
	females		0 5	20	0 9	4 3
dw/Jackson	8 weeks		25 5	178	7 0	61
controls	adult	8	1 9	25	1 0	13 7
	males/		0 7	9 6	0 4	5 2
	females					

along the same line and that the relative scatter along it is rather constant at all sulfate pool levels The incorporation values obtained in these experiments have been published elsewhere (Herbai *et al* 1970)

Strain differences in sulfate uptake and pool size

Table VII shows a comparison between N M R I mice lean litter mates to obese hyperglycemic mice and normal litter mates to dwarf mice with regard to the size of sulfate pool and the sulfation activity of cartilage The sulfate pools and likewise the incorporation rates varied markedly between the different strains studied at similar ages The young N M R I incorporated at a faster rate than the older ones expressed per rib cage The lengths of the cartilaginous ribs do not decrease with age (Herbai 1970b) and their thickness increases Thus the presented sulfate uptake values underestimate the differences in incorporation activity per unit weight

Discussion

Quantitative studies of sulfate incorporation into different tissues of living animals have been handicapped by the fact that sensitive micromethods for determination of sulfate in biological fluids have not been available With very few exceptions previous studies concerning sulfate incorporation into mucopolysaccharides (see references in

the Introduction) were carried out without knowledge of the size of the miscible sulfate pool of the animal. Hence, real calculations of the sulfate incorporation rates could not be carried out. Recently, a sensitive micromethod for sulfate has been described (Braselmann 1965) which permits determination of SO_4 in μg amounts with a precision of $\pm 0.2 \mu g$. Thus 1—2 μg can be determined with satisfactory precision. The method has been used for studies of ^{35}S incorporation into mucopolysaccharides of aorta (Kunz *et al.* 1964). Despite its sensitivity the method would not be sufficient for studies in small mice. Miller *et al.* (1961) described a method which is based on the use of radioactive barium as precipitating agent for plasma sulfate. It needs about 1 ml plasma. Thus for use in really small laboratory animals the present method of pool estimation can still not be supplanted by a direct measurement of specific activity of the circulating sulfate.

The present method does not require any blood sample. Since it is based on a double isotope assay the amounts of ^3H phenol and ^{35}S sulfate are not critical. The fact that a rapid *i.c.* injection and one squeezing of the urinary bladder are the only practical steps to be carried out in each animal makes serial work possible and 40 animals in a simultaneous experiment can be used without difficulty.

Since phenyl sulfate formation is a basic reaction in most species (Williams 1959) the method can probably be adapted to sulfate pool determinations in many different animal species. Fish may be an exception since is has been established that all injected phenol is excreted unchanged in free form very rapidly in gold fish (Boni and Iannaconne 1965).

Among the compounds studied for sulfate trapping ability phenol seemed to be the drug of choice for the following reasons. It is conjugated and excreted rapidly. It yields a readily separable and distinct sulfate conjugate after doses which elicit no toxic manifestations in the animals. Phenol in the necessary dosage does not interfere with the sulfate incorporation into cartilage. The frozen ^3H phenol stocks showed no signs of radiolysis.

The carrier phenol dose has to be high enough to overcome the influence of the endogenous phenol pool upon the $^3H/^{35}S$ ratio. It is also important to keep the phenol dose well below the lethal dose which has been estimated to about 125 mg/kg in the mouse (Spector 1956). The standard phenol dose used corresponds to about 1/7 of the *s.c.* minimum lethal dose for the mouse.

When the size of the non radioactive quenching dose of sulfate was determined the highest possible non toxic sulfate supply was aimed at. According to previous reports *i.c.* infusions of sodium sulfate solutions were tolerated in much higher amounts in the form of hypertonic (optimal tonicity 0.25 *M*) than of isotonic solutions (Ravasini and Martini 1933). The quenching dose of sulfate used in the present study was therefore given as a 0.25 *M* solution. The usual quenching dose per animal corresponds to about 24 mg SO_4 ions. This means a supply of sulfate which exceeds the normal sulfate pool of the mouse about 70 times. The dose is about 25 % of the *i.c.* minimum lethal dose for the mouse (Spector 1956).

The different experiments concerned with the development of the most suitable

standard method were described in detail and have been partly discussed in the Results As favourable aspects of the method can be mentioned that neither the estimations of the sulfate pools nor the incorporation rates are affected by the following conditions 1 The injected amounts of phenol and sulfate 2 The volume of the injection mixture 3 The exact time for taking the urine sample 4 The volume of the urine sample (one drop is sufficient) 5 The amount of silica gel scraped from the TLC plate 6 The amount of quenching sodium sulfate 7 Moderate variations in time between the sulfate quenching and killing the animals 8 The carefulness of the cartilage cleaning procedure (the intercostal muscles are practically free from radioactivity at the time of killing) and 9 The length of the storage time of urine and cartilage samples before processing and counting

After the rather short (30 min) incorporation period the animals were kept alive for about 4 hrs following the quenching since it has been observed that the interanimal variations for incorporation rate could be markedly reduced if the animals were alive for a couple of hours after sulfate quenching (Herbai unpublished observations) An explanation could be the apposition of non radioactive mucopolysaccharides which could protect the freshly formed ^{35}S tagged tissue during the stripping procedure

In most previous studies the sulfate incorporation rates into the tissues were expressed on a mg basis Since autoradiographic studies have recently detected profound regional differences in sulfate incorporation activity of costal cartilage in growing animals (Herbai 1970a), an expression of sulfate uptake per an anatomically well defined portion of the cartilage (Fig 2) has been used in all experiments

Recent investigations demonstrated marked circadian rhythm variations of the ^{35}S sulfate uptake into cartilage in mice (Simmons 1968) The present experiments were all carried out at the same time of the day beginning at 9 00 *a m*

Considerable variability has been reported in physiological and pharmacological parameters between different mouse strains (Brown 1965) Our findings regarding the differences in pool size between the investigated mouse strains and the pronounced strain variations in rates of sulfate uptake are a further example

I wish to express my gratitude to Professor E Barany and to docent L Terenius for their stimulating advice and criticism and to Mrs B Jansson for skilful technical assistance Thanks are also due to docent I Ekstedt for construction of the computer program

This work was supported by the Medical Faculty of the University of Uppsala and by the Nordisk Insulinfond

References

ALMQVIST S Studies on sulfation factor (SF) activity of human serum. Evaluation of an improved method for the *in vitro* bioassay of SF and the effect of glutamine and human growth hormone in the system *Acta endocr* (Kbh) 1961 *36* 31—50

BERNTSEN E Epiphyseal growth zones in acute lathyrism Uptake and distribution of $^{35}SO_4$ in cartilage and bone *Proc Soc exp Biol* (N Y) 1967 *124* 731—734

BERNTSEN E Epiphyseal growth zones in oestradiol treated rabbits *Acta endocr* (Kbh.) 1968 *57* 69—80

BONI P and A IANNACCONE Biotransformazione eliminazione e tossicita acuta del fenolo nei pesci *Lo Sperimentale* 1965 *115* 71—81

BOSTROM H and A VESTERMARK Some aspects on sulphate conjugation *Biochem Pharmacol* 1961 *6* 72—81

BOSTROM H B E GUSTAFSSON and B WENGLE Studies on ester sulphates 18 Ester sulphate formation in the germfree rat *Proc Soc exp Biol* (N Y) 1963 *114* 742—747

BOSTROM H K BERNTSEN and M W WHITEHOUSE, Biochemical properties of anti inflammatory drugs II Some effects on sulphate ^{35}S metabolism *in vivo* *Biochem Pharmacol* 1964 *13* 413—420

BOSTROM H Sulfate conjugation and conjugated sulfates *Scand J clin Lab Invest* 1965 *17* Suppl 86 33—52

BOYLAND E D MANSON and S F D ORR The biochemistry of aromatic amines 2 The conversion of arylamines into arylsulphamic acids and arylamine N glucosiduronic acids *Biochem J* 1957 *65* 417—423

P ... ivitat von Muko-

E ... influssung einiger
1968 *18* 801—
806

BROWN A M Pharmacogenetics of the mouse *Lab Animal Care* 1965 *15* 111—118

CHESLEY L C Growth hormone activity in human pregnancy II Assays with an *in vivo sulfation method* *Amer J Obstet Gynecol* 1963 *87* 8—12

COLLINS E J and V F BAKER Growth hormone and radiosulfate incorporation I A new assay method for growth hormone *Metabolism* 1960 *9* 556—560

DAUGHADAY W H and D M KIPNIS The growth promoting and anti insulin actions of somatropin *Recent Progr Hormone Res* 1966 *22* 49—99

DENKO C W and D M BERGENSTAL The effect of hypophysectomy and growth hormone on S^{35} fixation in cartilage *Endocrinology* 1955 *57* 76—86

DZIEWIATKOWSKI D D Effect of thyroxine and thiouracil on ^{35}S deposition in articular cartilage *J biol Chem* 1951 *189* 717—727

FRIBERG U and N R RINGERTZ Uptake of radiosulphate and radiophosphate in various

Gc ... keletal

Hı ... ns tive
58

HERBAI G Incorporation and disappearance of sulfate in different regions of mouse and rat costal cartilage *in vivo* and *in vitro* *Acta physiol scand* 1970b *79* 541—551

HERBAI G S WESTMAN and C HELLERSTROM The growth hormone dependent incorporation of sulphate into the costal cartilage of obese hyperglycaemic mice of different ages *Acta endocr* (Kbh) 1970 *64* 415—420

KHOKHAR S A and D M HILKER Decreased (^{35}S) sulfate uptake into aorta and liver of rats given histamine injections Lack of effect of a high fat diet *J Atheroscler Res* 1967 *7* 767—770

KOVACS I B P GOROG L SZPORNY and G FEKETE Effect of vitamin K on connective tissue metabolism *Biochem Pharmacol* 1967 *16* 575—578

... Sul

... A J

... es to measure body fluid constituents I Plasma sulfate *J Lab Clin Med* 1961 *58* 656—661

MURPHY W R W H DAUGHADAY and C HARTNETT The effect of hypophysectomy and growth hormone on the incorporation of labeled sulfate into tibial epiphyseal and nasal cartilage of the rat *J Lab clin Med* 1956 *47* 715—722

PINKERTON W and M WEBBER A method of injecting small laboratory animals by the ophthalmic plexus route *Proc Soc exp Biol* (N Y) 1964 *116* 959—961

PRIEST R E R M KOPLITZ and E ... nd oac tive sulfate into cart lage and aortas ...

RAVASINI G e L MARTINI Richerche ... iez oni endovenose di soluzioni iper ... ed ... 13 33 67—91

SALMON W D JR and W H DAUGHADAY A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage *in vitro* *J Lab clin Med* 1957 *49* 825—836

SALMON W D JR P H BOWER and E Y THOMPSON Effect of protein anabolic steroids on sulfate incorporation by cartilage of male rats *J Lab clin Med* 1963 *61* 120—128

and London

TOVE, S B, F H SMITH, C T YOUNG and F W SHERWOOD, Effect of source of dietary protein on the unsaturated fatty acids in the carcass fat of the rat *J Nutr* 1954 *54* 49—57

[illegible] RAVE, *Untersuchungen* [illegible] wechsel der sulfatierten [illegible]—12

[illegible] *Wirkung von Aurothio-* [illegible] des Bindesgewebes *Z Rheumaforsch* 1969 *28* 287—298

[illegible] 1959 pp 278—

[illegible] ondroitin schwef- [illegible]

[illegible] plasma. Brain and liver of rats after castration and sex hormone administration *Acta endocr* (Kbh.) 1964 *16* 12—24

WUSTEMAN, F S, K S DODGSON, A G LLOYD, F A ROSE and N TUDBALL, Thin layer chromatography in the study of ester sulphates *J Chromatogr* 1964 *16* 334—339

ZUMEL, M C L., Estudio biocientico de la sulfoconjugacion por radio-trazadores y su posible aplicacion a procedimientos de diagnostico *Arch Inst Farmacol exp* (Madr) 1965 *17* 145—165

Acta physiol. scand. 1970. 80. 492—501

From the Department of Physiology, Royal Veterinary College, Stockholm Sweden, and the Department of Physiology, Yale University School of Medicine, New Haven, Conn, USA

Synchronization of Activity in Afferent Nerve Branches within the Frog's Muscle Spindle

By

D. OTTOSON and G. M. SHEPHERD

Received 29 April 1970

Abstract

OTTOSON, D. and G. M. SHEPHERD. *Synchronization of activity in afferent nerve branches within the frog's muscle spindle.* Acta physiol. scand. 1970. 80. 492—501.

The mechanisms underlying the generation of activity in the afferent nerve branches within the muscle spindle have been studied in isolated frog spindles. Repetitive stimulation at given frequencies and strengths caused a disintegration of the synchronous activity of the spindle as revealed by a decomposition of the impulse into small abortive spikes of different amplitudes. The breakdown of synchronous activity was preceded by a stage during which the impulse appeared gradually later but then remained steady at a given delay. The isolated receptor potential exhibited a latency shift closely similar to that of the impulse response under repetitive stimulation. Treatment of the spindle with tetrodotoxin at critical concentrations caused a decomposition of the impulse similar to that with repetitive stimulation. It could be shown in these spindles that synchronization of the activity within the spindle is related to the amount of depolarization of the sensory endings. The mechanisms for generation of activity in the spindle are discussed in relation to the structural organization of the branches of the afferent fibre. It is concluded that the anatomical and functional properties of the spindle provide the basis for a synchronization of the activity of its different parts during the response to an applied stretch.

The frog's muscle spindle is innervated by a single afferent nerve fibre. Within the capsule of the spindle this fibre gives rise to a system of myelinated branches which ultimately terminate in fine sensory chains. The chains are unmyelinated and consist of small nerve bulbs connected by thin tubes or links. Katz (1961) estimated the number of sensory bulbs in one spindle to be about 10 000, i. e., an average of 100 in series along 100 parallel lines which all are ultimately connected to the one afferent nerve fibre.

The structural complexity of this branching system raises the question of whether activity in the branches is asynchronous or synchronous. In recordings of the spontaneous impulse discharge in slack spindles held under little tension Katz (1950) observed small monophasic potentials alternating with the large action potentials. He identified these small potentials with abortive impulses generated in single nerve

branches by restricted groups of sensory terminals When the spindle was stretched the small potentials merged with the large action potentials of the afferent nerve discharge and disappeared We have made similar observations in the isolated spindle preparation In addition it has been found that under certain experimental conditions the large action potentials in the response to stretch can be decomposed into smaller units In this paper we report these experiments and discuss the functional properties of the spindle in relation to recent electronmicroscopical evidence about the structure of the spindle innervation The conclusion is made that with increasing stretch of the spindle the nerve branches tend to be active in synchrony

Methods

of the nylon rods was attached through a metal lever to a loudspeaker coil (Philips AD 2300 BZ) Stretches were applied by driving the loudspeaker coil with electrical pulses The move

Japan

Results

Latency change and failure of impulse The response of the muscle spindle is characterized by a high reproducibility as shown by the fact that the spindle may continue to give almost identical responses to a given stimulus in experiments lasting for hours However, when the spindle is subjected to very strong stretches or to streches at high frequencies changes of the response appear which may lead to a decomposition of the impulses into smaller unitary activity The changes preceding the disintegration of the regular response are characterized by an increasing delay of the individual spikes and under critical conditions a failure of the spikes to appear

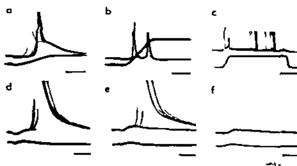


Fig 1 Latency shift of impulse response of spindle during repetitive stretches Record *a* brief stretch at 15/s giving a single impulse *b* stronger stretch at 10/s giving two dynamic spikes *c* steplike stretch at 6/s giving two dynamic and two static spikes Records *d—f* from another spindle) gradual delay and final failure of impulse elicited by brief stretch at 100/s Time bars in *a* 5 msec in *b* 10 msec in *c* 20 msec in *d—f* 2 msec

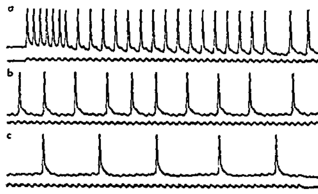
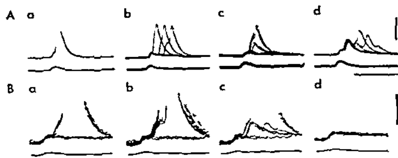


Fig 2 Continuous recording of impulse response to brief repetitive stretches at 300/s Time bar 25 msec

The superimposed recordings in Fig 1 *a* show the gradual delay of a spike elicited by brief linearly rising stretches repeated at 15/s With the first few stretches the spike appeared gradually later but then remained steady at a given latency for the following stretches The generation of the impulse response at a fixed delay was also seen when there were two spikes during the dynamic phase of stretch (*b*) although the timing of the second spike was less rigid than for the first impulse A similar effect was also seen during static stretch (*c*) With weak stretches of short duration the delay also increased and the spike tended to arise at one or several latency modes (*d*) As the repetitive stretch continued the spike began to drop out (*e*) and ultimately the underlying receptor potential was obtained in isolation (*f*) (*cf* Fig 5)

The gradual increase in latency and final failure of the spike to appear was studied in more detail by recording the responses on moving film as is shown in Fig 2 In this experiment the spindle was stimulated with a brief stretch repeated at 300/s For the first seven stretches the spindle responded with a spike for each stretch In the following period only every second stretch produced a spike The stretches which failed to elicit an impulse produced small potential elevations As the repetitive stretches continued there was a progressive lengthening of the periods during which only the small potentials appeared

Disintegration of spike Repetitive stretching at given strengths and frequencies caused a disintegration of the action potential into small abortive spikes A typical example of this is illustrated by the records *a*—*d* in Fig 3 A Record *a* shows the spike elicited by a single brief stretch It may be noted that the spike had a notch at its foot indicating the takeoff of the spike from the underlying receptor potential During a brief repetitive train (*b*) of the same stretch the spike arose gradually later and another notch appeared on the rising phase of the spike Later in the stimulation period the spike failed to appear and only the receptor potential was obtained With repetitive trains of the same stretch at higher frequency (*c*) the second component of the spike dropped out and later also the first component After intense trains still further splitting up of the response could be obtained as shown in *d* It can be seen that the response consisted of a sequence of at least four different potential elevations superimposed on the receptor potential These elevations would appear to



[illegible]

represent conducted activity in different branches of the nerve fibre since they are of different amplitudes and do not affect each other by refractoriness The records in Fig 3 *B* are from another spindle Repetitive stimulation led to a failure of the main spike and a small abortive spike was obtained (*c*) Further stimulation led to complete failure of the conducted activity and the isolation of the underlying receptor potential (*d*)

The gradual decomposition of the single impulse during repetitive stretches can be followed in detail in Fig 4 In this experiment the spindle was subjected to brief stretches repeated at a frequency of 150/s In the beginning of the train of stimuli each stretch produced a full spike As stimulation continued the spike began to break up into several smaller subunits which appeared alternating with small potential elevations

From the results of the above described experiments it appeared that prolonged repetitive stimulation leads to a progressive breakdown in the mechanisms of conduction in the branches of the afferent nerve This effect could be attributed to a prolonged refractoriness and to blockage of the activity of the branches (see Discussion)

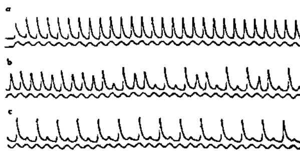


Fig 4 Gradual disintegration of spindle impulse into smaller sub-units Continuous recording of response to brief stretch at 150/s Time bar 25 msec

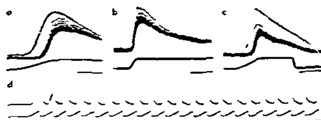


Fig 5 Effect of repetitive stretching on latency and amplitude of receptor potential. Records *a*—*c* show superimposed responses to stretches of different parameters at 20/s (*a*) and 10/s (*b* and *c*) Time bars in *a* 5 msec, in *b* and *c* 10 msec. Record *d* shows continuous recording of response to brief dynamic stretch at 30/s. Time bar 50 msec

Changes of receptor potential under repetitive stimulation In order to study to what extent the above described changes of the impulse response might be attributed to changes of the receptor potential, the effect of repetitive stretches was studied in spindles treated with lignocaine so that only the receptor potential was obtained The records *a*—*c* in Fig 5 show superimposed recordings from one of these experiments. As can be seen there was a shift in latency and a time locking of the receptor potential to a fixed delay closely similar to the corresponding changes seen in the impulse records (*cf* Fig 1) It may also be noticed that there was a reduction in height of the dynamic peak of the response while the static phase of the potential was relatively unaffected (*b* and *c*) The gradual change in amplitude of the dynamic potential is also illustrated in *d* which shows continuous recordings of the responses produced by brief phasic stretches repeated at 30/s. At this frequency the intervals between the stretches were long enough to allow the potential to return to near zero

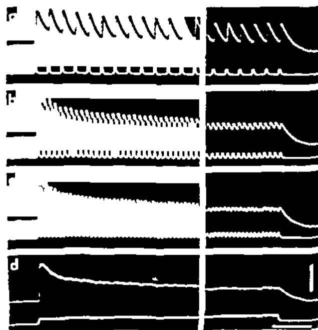


Fig 6 Summation of receptor potentials with increasing frequency of stretch Brief stretches of same amplitude at 100/s (*a*) 150/s (*b*) 200/s (*c*) and 400/s (*d*) Time bar 100 msec Vertical bar 1 mV

after each stretch When stretches were repeated at higher frequencies the responses summated (Fig 6) With a sufficiently high frequency a response was obtained which in its general pattern was strikingly similar to that obtained with a steplike stretch (Fig 6*d*)

The above described results show that the transducer action of the spindle is a remarkably reproducible event even during rapid repetitive stretch The main changes produced by repetitive stretching at high rates is a delay of the response and a reduction in amplitude These changes may be responsible for the characteristic latency changes of the impulse response and may also contribute to the failure of the impulse as illustrated in Fig 2 The desynchronization appears to be the result of a breakdown of the conducted activity in the branches

Synchronization of conducted activity during stretch As described by Katz (1950) the abortive conducted activity may be seen when the spindle is held at resting length However, in our experiments small spikes were also seen occasionally when the spindle was stretched It could then be shown that their mergence into the large conducted impulse of the stem fibre was dependent on the strength of the applied stretch, as illustrated by the recordings in Fig 7 The spontaneous discharge of the spindle in *A* was characterized by alternating large and small spikes When the spindle was subjected to a slowly rising stretch the small spikes gradually merged into the large spike and disappeared during the static phase of stretch This suggests that the synchronization of the activity of the individual branches of the afferent fibre is a gradual event, the determining factor being the amount of depolarization of the end ings Another evidence for this is produced by the experiment illustrated by the records in *B* of Fig 7 The spindle was treated with tetrodotoxin at a concentration just strong enough to block the response to a brief threshold stretch As the strength of the stretch was increased a small spike first appeared (*a*) and then a second (*b*) With further increase these two spikes merged into a typical single main spike (*c* and *d*) The merging of the two spikes appears to be continuously graded there is no effect of refractoriness of the first spike on the second



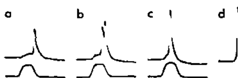
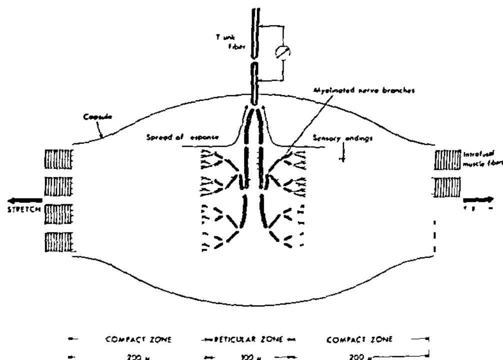


Fig 7 Synchronization of activity within branching tree of spindle with increasing amount of stretch *A* effect of slowly rising stretch Note that small abortive spike merges with the main impulse as stretch increases *B* effect of increase in amplitude of brief stretch on response of spindle treated with tetrodotoxin at concentration of 2×10^{-8} g/ml Time bars in *A* 100 msec in *B* 20 msec

Discussion

The experiments illustrated in Fig 7 provide evidence that activity in the branches of the nerve becomes synchronized with increasing stretch of the spindle and *pari pa su* increasing depolarization by the receptor potential We conclude that with increasing stretch, differences in time course of activation of different regions of the nerve branches are counteracted by the receptor potential This implies a substantial and relatively uniform spread of the receptor current through the branching system. There is independent evidence of this spread in the relatively large receptor potentials recorded from the trunk fibre The physiological results therefore imply that in the response to stretch the nerve trunk, branches and sensory terminals are in close functional relation This raises the question how such a relation is compatible with structural complexity of the branching system mentionned in the Introduction



a given order in comparison with figures given by Katz 1961) In the ... 100 parallel rows of 50 end bulbs on each intrafusal fibre giving a total for the four fibres of 1000 end bulbs Katz 1961 estimated a total of 10 000 end bulbs for one spindle which could be obtained with a model made of 10 parallel rows of 100 end bulbs on each of 10 intrafusal fibres. The branching system for such a model would be prohibitively complex to show even in diagrammatic form. The model in Fig 8 can therefore be considered as a conservative approximation for a small spindle For further details see text

Recent anatomical studies permit a fairly precise reconstruction of the branching tree of the afferent nerve which helps resolve this question The schematic diagram of Fig 8 is based on the electronmicroscopical studies of Katz (1961) and Karlsson Andersson Cedergren and Ottoson (1966) The latter note that An immense ramification (of myelinated nerve branches) must occur at the transition zone between the reticular and sensory compact zone to account for the number (of sensory nerve endings) encountered These authors suggested that three to six orders of branching could account for the number of endings observed depending on whether branching was dichotomous or trichotomous The case of dichotomous branching is illustrated by the branching tree model in Fig 8

The spatial extent of this branching tree in Fig 8 has been estimated from the following evidence The linear extent of the reticular zones of the intrafusal fibres is approximately 100 μ (Katz 1961 Karlsson *et al* 1966) the diameter of the capsular region of the isolated spindle is about 100 μ (Ottoson and Shepherd 1968 1970) The majority of the chains of sensory nerve endings arise from preterminal myelinated branches near the zone of transition between reticular and compact zones (Karlsson *et al* 1966) and it is therefore reasonable to assume that the radial extent of the tree of myelinated nerve branches in relation to the stem fibre is very short of the order of 100 μ The fact that the nerve branches are not only short but also myelinated provides the structural substrate for the close functional relation between the sensory endings and the stem fibre Elsewhere Ottoson and Shepherd (1969) have estimated that the electrotonic characteristic length for the equivalent cylinder' (Rall 1962) representing the tree is of the order or 1 or less In view of the fact that under microscopic observation the spindle stretches like an uniformly elastic element (Ottoson and Shepherd 1968 1970) it has been suggested that during stretch there is a relatively uniform depolarization of sensory endings throughout the spindle This would contribute to the synchronization of activity in the different branches and also provide for summing of the activity within the chain of sensory end bulbs to give large depolarizations

In view of these considerations the diagram of Fig 8 may be used as a framework for discussing the probable mechanisms of impulse generation in the spindle under different experimental conditions In the resting slack spindle we assume with Katz 1950) that single preterminal branches or restricted groups of them are capable of spontaneously generating action potentials which are recorded as miniature spikes As Katz pointed out such a spike could raise the excitability of other nearby branches by electrotonic spread and could sum with other spikes to build up the propagated impulse in the trunk fibre Apparently the spontaneous discharge of the afferent fibre depends mainly on the one terminal or group of terminals with the highest safety factor for conduction through the branching tree

The resting spindle is on the verge of firing as shown by its spontaneous discharge and the fact that its threshold to stretch is extremely low Therefore in the response to a slow weak stretch the initial impulse is set up by a very weak stimulus and consequently a very small receptor potential Katz (1950 suggested that the first im

pulse is generated when the receptor depolarization may be just sufficient to trigger the most excitable terminal or terminals Our results in the isolated spindle are consistent with that interpretation for very low levels of stretch However all impulses subsequent to the first are generated on a background of larger depolarization by the sensory terminals We have pointed out above that this depolarization reaches considerable magnitude that it is probably relatively uniform in different parts of the spindle and that it spreads with little delay and only moderate decrement through the branches to the trunk fibre Our results indicate that the effect of this receptor depolarization is to cause the near synchronous generation of conducted activity throughout the branching system of the afferent nerve

An alternative possibility for the generation of the impulse discharge in response to stretch is that an impulse which first arrives at a branch point spreads into the other branches and blocks their activity by antidromic invasion Evidence for such a mechanism has been obtained for tactile receptors in the skin (Lindblom and Tapper 1967) We do not rule out such a mechanism in the generation of the spontaneous discharge in single preterminal branches in the slack spindle (see above and Katz 1950) and possibly also in the generation of the initial impulse at very low levels of stretch However for stretches giving appreciable receptor potentials our results indicate that there is normally a near synchronous activity of the nerve branches The extreme shortness of the branches and the spread of the receptor potential throughout the branching tree to the first node of the afferent fibre are essential conditions which differentiate response generation in the spindle from that in the skin receptors

When stretch is applied rapidly the time between the onset of stretch and the onset of the receptor potential is of the order of several hundred microseconds (Shepherd and Ottoson 1965 this includes the time for transduction of the mechanical stimulus as well as spread of the resulting receptor potential through the branches to the recording site in the afferent fibre There is a similar short delay between the onset of the receptor potential and the foot of the initial spike Therefore when onset of stretch is sufficiently rapid the first impulse in the response appears also to be generated by a near synchronous action of the branching tree Even when there is a degree of asynchrony as produced by repetitive stretches (Fig 3 *A d*) or administration of tetrodotoxin (Fig 7 *B* there is no observable effect of refractoriness of one fractional potential on another This is consistent with Katz (1950) conclusion that a miniature spike in one branch does not render other branches refractory

It may be concluded that under most conditions of stretch there is at the first node of the trunk fibre a confluence of the current flow from activity through ut the sensory tree At low levels of stretch the depolarization due to the receptor potential is small and the discharge in the trunk fibre will be expected to arise mainly from the conducted activity fed into it from the branches Under these conditions desynchronized activity may sometimes be seen (*cf* Fig 7 *A* With higher levels of stretch the receptor potential reaches higher amplitudes the effect of this is to promote synchronous action of the branches as discussed above and it may also tend to shift

the site of impulse generation toward the first node of the trunk fibre It may be speculated that the actual site of impulse initiation could depend on several factors 1) a possible lower threshold of the trunk fibre, 2) the slower recovery time of the finer nerve branches (Ottoson and Shepherd 1969), 3) the gradient of depolarization through the branching tree due to the receptor potential, and 4) a possible inactivation of impulse generation in the branching tree with increasing depolarization at higher rates and levels of stretch

This work has been supported by grants from the Swedish Medical Research Council projects no B69 14X 43 05 A and B70 14X 43 06 B

We also wish to thank Miss Olga Popoff for technical assistance

References

HAAPANEN L E A high sensitivity capacitance meter *Electron Engng* 1962 *34* 183—185

KARLSSON, U E ANDERSSON CEDERGREN and D OTTOSON Cellular organization of the frog muscle spindle as revealed by serial sections for electron microscopy *J Ultrastruct Res* 1966 *14* 1—35

KATZ B Action potentials from a sensory nerve ending *J Physiol* (Lond) 1950 *111* 248—260

KATZ B The terminations of the afferent nerve fibre in the muscle spindle of the frog *Phil Trans B* 1961 *243* 221—240

LINDBLOM U and D M TAPPER Terminal properties of a vibrotactile sensor *Exp Neurol* 1967 *17* 1—15

OTTOSON D The effect of acetylcholine and related substances on the isolated muscle spindle *Acta physiol scand* 1961 *53* 276—287

OTTOSON D [illegible]

O [illegible]

O [illegible]

C [illegible]

RALL W Theory of physiological properties of dendrites *Ann N Y Acad Sci* 1962 *96* 1071—1092

SHEPHERD G M and O OTTOSON Response of the isolated muscle spindle to different rates of stretching *Cold Spr Harb Symp quant Biol* 1965 *30* 95—103

Acta physiol. scand. 1970, 80. 502—509

From the Department of Pharmacology and Institute of Medical Chemistry, University of Uppsala, Sweden

Regional Differences in the Incorporation Rates of ^{3}H-Acetate and ^{35}S-Sulfate into Chondroitin Sulfate of Mouse Costal Cartilage in Vitro

By

GEORG HERBAI and ULF LINDAHL

Received 4 May 1970

Abstract

HERBAI, G and U LINDAHL *Regional differences in the incorporation rates of ^{3}H acetate and ^{35}S sulfate into chondroitin sulfate of mouse costal cartilage in vitro* Acta physiol scand 1970 80 502—509

The rates of incorporation of ^{3}H acetate and ^{35}S sulfate into chondroitin sulfate of mouse costal cartilage were studied by an *in vitro* incubation technique Cartilage specimens from two different regions of costal cartilage prepared from growing mice, were incubated for varying periods of time Chondroitin sulfate was isolated after proteolytic digestion of the tissues by repeated precipitation with cetylpyridinium chloride The average incorporation rates which were followed during 24 hrs, showed a marked decrease after 2 hrs of incubation and then remeained essentially constant During the entire incubation period much more exogenous sulfate than acetate was incorporated into the segments Considerably more exogenous sulfate and acetate were incorporated into the lateral segments of the cartilage including the osteochondral junctions than into the medial segments It is concluded that chondroitin sulfate is synthetized at a higher rate in the vicinity of the osteochondral junction than in other portions of mouse costal cartilage

A large amount of experimental work has been devoted to clarify the mechanisms of biosynthesis and metabolism of glycosaminoglycans in several kinds of connective tissue from different species Considerable advances have been made in understanding how these polysaccharides are synthetized and a voluminous literature has accumulated, much of which has been lately reviewed (Boström and Rodén 1966 Silbert 1966 Dodgson and Lloyd 1968 Muir 1969)

The mode of regulation under physiological conditions of the biosynthetic process is unclear Very little is known of how the glycosaminoglycan producing cells are affected by variations in the properties of the extracellular environment Furthermore, the experimental approach to this problem is complicated by the difficulties in determining, in intact cells or tissues the actual rates of polysaccharide synthesis As part of a more extended study of the factors involved in the regulation of glycos-

aminoglycan biosynthesis, the present investigation has been aimed at determining the rates of chondroitin sulfate synthesis in different types of cartilage In previous studies marked regional differences in ³S sulfate uptake of different parts of mouse and rat costal cartilage have been found by an autoradiographic technique (Herbai 1970a) and by quantitative analysis of sulfate uptake (Herbai 1970b) The osteochondral junction of the rib exhibited much higher sulfate incorporation than did the remaining parts of the costal cartilage both *in vivo* and *in vitro* Since the results of these studies were expressed on a dry weight tissue basis it was of interest to investigate, whether the observed regional differences would also apply to the rate of incorporation of labeled precursors into chondroitin sulfate

Materials and Methods

Materials

Growing female N M R I mice weighing about 20 g were used throughout the study Their age was about one month In one experiment involving the determination of the chondroitin sulfate content of dried cartilage a group of six months old mice were also included All animals were obtained from Anticimex AB Sweden They were housed in plastic cages and had free access to pelleted food (Anticimex no 213) and tap water Constant temperature and day

fic activity 500

nsion) was pur

Analytical methods

he method f R and M 1962 The el cos

barium acetate (2 7 V/cm for 6 hrs) (Wessler 1968) or in 0 1 M HCl (1 9 V/cm for 2 hrs) (Wessler 1970)

The radioactivity of polysaccharide samples doubly labeled with ^{35}S and ^{3}H was determined in a Packard Model 3324 liquid scintillation spectrometer Aliquots of 0 1 ml were mixed with 10 ml of scintillator solution (Herbai 1970b) in counting vials which were then kept in the spectrometer for 4 hrs under refrigeration prior to analysis Each sample was then counted until at least 20 000 counts had accumulated in the tritium channel The channel overlap from the ^{35}S to the ^{3}H channel was 30 % Automatic external standardization was used for quench correction The calibration factor of the spectrometer was determined by means of standardized reference solutions of ^{3}H hexadecane and lithium ^{35}S sulfate obtained from the Radiochemical Centre Amersham the counting efficiency for ^{35}S was 30 % and for ^{3}H 12 % All results were adjusted to 100 % efficiency (DPM) and could thus be expressed as picomoles of acetate and sulfate incorporated from the medium per µmole of chondroitin sulfate repeating disaccharide unit

Incubation experiments

The animals were killed with ether and their chest cages were dissected free *in situ* An anatomically well defined portion of the rib cage was cut out from each animal (Fig 1) Lateral and med l egment of

ustrated in Fig

ach region was

enty *regional*

e combined in

1/2 mm long

pieces with a scalpel prior to incubation however some incubations were performed with intact or with homogenized cartilage segments In the latter case the tissue was treated, in the

STERNUM
III
IV
V
VI
VII
LATERAL ZONE
MEDIAL ZONE
X PHOID
CARTILAGE
BONE
OSTEOCHONDRAL JUNCTION

Fig 1 Schematic representation of part of the cartilaginous portion of the mouse rib cage dotted lines indicate how the cartilage was cut in order to yield ten homologous tissu ments (length 4 mm) from the lateral ("active") and the juxta sternal medial ("rest zones Lateral segments included also the osteochondral junctions and a small piece of adj bone

incubation medium, with a Polytron® homogenizer (Kinematica, GMBH, Schweiz) ope at 8 000 R P M for 1 min The incubations were carried out in stoppered 50 ml Erlenr flasks containing 5 ml of Krebs Ringer phosphate solution, which was prepared accordi ept that magnesium sulfate was chang on D Glucose was added to a final co n was 0 1 mM with respect to both so ^{3}H-acetate and 5 μC of ^{35}S sulfate pe The relative proportions of the two labeled compounds were chosen to obtain optimum rability of the ^{3}H ^{35}S-mixture After saturation of the incubation mixtures with oxyge *flasks were shaken at 37° C for varying periods of time (0 5—24 hrs) At the end of* incubation period the reaction was stopped by immersion of the flasks into a boiling water for 10 min Thereafter the vials were kept frozen at −20° C until the papain dige procedure

Glycosaminoglycans were isolated from the incubation mixtures by precipitation with c pyridinium chloride (CPC) following proteolytic digestion of the cartilage slices (Scott 19 The content of each vial was mixed with 1 2 ml of 0 5 M acetate buffer, pH 5 5 contai 1 0 M NaCl 0 05 M cystein HCl and 0 05 M EDTA After the addition of 25 μl of pa suspension the digestions were allowed to was clarified by passage through a small p was subsequently extracted with 2 ml of t extract were added 2 ml of 1 % CPC in centrifugation The precipitate was washed with solved in 1 0 of 2 M and 1 0 ml of 1 % complex was dissolv the solution was kep with CPC from solutions of low ionic strength as described above and was finally conve to the sodium salt by precipitation with 4 volumes of ethanol from 2 M NaCl The precipi was washed with ethanol dissolved in 0 2 ml of water and analyzed for radioactivity and ur acid

Results

Electrophoresis of the isolated glycosaminoglycan preparations showed the presen of a single major component which had migrated like chondroitin 4 sulfate in bo 0 1 M hydrochloric acid and in 0 1 M barium acetate Galactosamine was the on

TABLE I. Yields of chondroitin sulfate from lateral ("active") and medial ("resting") segments of

	Young growing mice		Adult mice	
	lateral zone	medial zone	lateral zone	medial zone
Yield of chondroitin sulfate (mg uronic acid per 100 mg of dry cartilage)	3 4	3 0	2 4	2 4

amino sugar detected by gas-liquid chromatography, thus demonstrating the complete separation of chondroitin sulfate from any keratan sulfate originally present in the tissue A comparison between the polysaccharide contents, on a dry-weight tissue basis, of lateral ("active") and medial ("resting") cartilage segments showed no significant differences within each age group However, non-growing adult mice exhibited lower polysaccharide contents in both types of rib cage segments than did young growing animals (Table I) The experiments reported below were all performed with cartilage from young growing mice

Preliminary incubation experiments showed that maximal incorporation of sulfate and acetate into chondroitin sulfate occurred with diced cartilage, the incorporation obtained with either whole cartilage segments or with homogenized tissue was considerably lower (Table II), in agreement with the previous observations of Bostrom and Månsson (1953) Diced cartilage was therefore used throughout the investigation The procedure employed in the isolation of chondroitin sulfate from the incubated tissue efficiently separated non-incorporated acetate and sulfate from the polysaccharide, as seen from the specific radioactivity of preparations isolated from cartilage incubated either at 0° C or at 37° C after boiling of the tissue (Table II)

TABLE II

	Diced cartilage	Whole cartilage segments	Homogenized cartilage	Diced cartilage incubated at 0° C	Diced cartilage boiled for 12 min prior to incubation
^{35}S incorporation Per cent of maximal	100	24	9	0 7	0 8
^{3}H incorporation Per cent of maximal	100	27	15	0 6	1 5

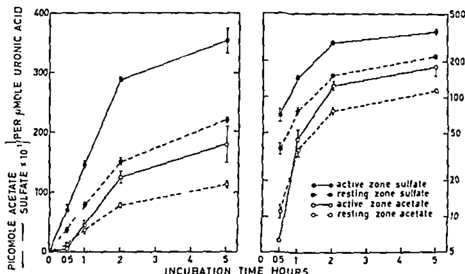


Fig 2 Time course of the *in vitro* incorporation of exogenous sulfate and acetate into chondroitin sulfate of mouse costal cartilage (lateral = active and medial = resting zones) The values are presented both as linear and as semilogarithmic plots Each symbol represents the mean of two experiments The range is indicated by vertical bars The cartilage material of each experiment consisted of twenty regionally homologous tissue segments collected from two animals

The time course of the incorporation of exogenous sulfate and acetate into chondroitin sulfate is illustrated in Fig 2 and in Table III The incorporation rates both ions were constant in both the lateral and medial zones between 0 5 and 2 hrs of incubation After this period the rates decreased considerably and then remained more-or-less unchanged for more than 20 hrs

The two zones of costal cartilage showed marked differences with respect to the rates of incorporation of sulfate and acetate into chondroitin sulfate the rates being about twice as high in the lateral (active ') as in the medial ('resting') zone (Fig

TABLE III Rates of incorporation from the incubation medium of exogenous sulfate and acetate into chondroitin sulfate of lateral (active) and medial (resting) zones of costal cartilage from growing mice

Incubation periods hours	Picomoles incorporated per μmole uronic acid × hour per				Relative incorporation rates lateral/medial zone		Molar ratio sulfate/acetate	
	lateral zone		medial zone					
	sulfate	acetate	sulfate	acetate	sulfate	acetate	lateral zone	medial zone
0— 1/2	1 420	13	740	22	1 9	0 6	113	34
1/2—1	1 510	77	830	52	1 8	1 5	20	16
1—2	1 425	81	730	41	1 9	2 0	18	18
2—5	217	18	232	12	0 9	1 5	12	20
5—10	411	32	229	19	1 8	1 7	13	12
10—24	229	30	156	28	1 5	1 1	8	6

2 Table III) For reasons indicated below this finding did not apply to the initial half hour period of acetate incorporation

Throughout the entire incubation period the amounts of sulfate transferred from the medium into chondroitin sulfate considerably exceeded those of acetate (Table III) The incorporation of labeled acetate showed an initial lag period whereas the incorporation of labeled sulfate was apparently linear during the same period as a result the sulfate acetate molar ratios shown in Table III were higher during the initial than during the later stages of the incubation The delay observed for the incorporation into chondritin sulfate of exogenous acetate was consistently more pronounced in the lateral than in the medial zone of costal cartilage as seen from the drastic change during the first hour of incubation of the relative acetate incorpora tion ra es of the two zones (Table III)

Discussion

Radioactive precursors particularly ^{35}S sulfate have been extensively employed both *in vitro* and *in vivo* as indicators of glycosaminoglycan biosynthesis Although much valuable information has been gained it has become increasingly evident that the quantitative evaluation of incorporation experiment involving whole cells offers several pitfalls A survey of the various factors which have to be taken into account includes A) the ability of precursor substances to penetrate into the cell B) the intracellular intermediary precursor pools with particular regard to the relation be tween different inflow routes to these pools C) the cell concentration in the tissue and the amounts of intracellular acceptors as related to the bulk of extracellular poly saccharide material and D) when ^{35}S sulfate is employed the degree of sulfation of the synthetized product It is obvious that experiments aimed at comparing the poly saccharide synthetizing ability of different tissues should be evaluated with con siderable caution

In an attempt at least partly to overcome the interpretation d fficulties outlined above the experiments performed in the present study involved the simultaneous incorporation into polysaccharide of two d fferent labeled precursors The results clearly show that the incorporation of both exogenous sulfate and acetate into chondroitin sulfate occurs at a more rapid rate in the lateral (active) than in the medial (resting) zone of mouse costal cartilage

In previous studies concerned with the chemical changes during calcification comparisons were made between resting and hypertrophic cartilage zones regard ing the contents of organically bound sulfur in calf epiphyseal cartilage (Weatherell and Weidmann 1963 Wuthier 1969) The results were in accord with those of the present study on mice ind cating that the dry weight concentrations of chondroitin sulfate in the active and resting zones are approximately similar Hence it seems reasonable to infer that the formation of new polysaccharide is more rapid as related to the pre-existing polysaccharide pool in the lateral (active) than in the medial (resting zone Thus the results of the present study apparently parallel those of Herbai (1970a and b) who observed regional differences both in aut

radiographs and on a dry-weight tissue basis, in the ability of mouse and rat costal cartilage to incorporate inorganic ^{35}S-sulfate The differences recorded in the quantitative studies, including the present one, are rather moderate, as compared to the autoradiographic patterns obtained with ^{35}S-sulfate (Herbai 1970a) This discrepancy should probably be ascribed to the fact that the lateral "active" cartilage segments contained considerable portions of "resting' tissue in addition to the narrow highly active osteochondral junction Thus the high rate of polysaccharide biosynthesis in the osteochondral junction would be "diluted' by the remaining "resting' portion of the sample Autoradiographic (Bélanger 1954) and electron microscopic (Schenk *et al* 1967 Matukas and Krikos 1968) investigations on the growth zone of calcifying cartilage demonstrated that this zone is histologically inhomogeneous Hence, the present results offer no conclusion as to the polysaccharide-synthetizing capacity of the individual cells, since the cell density of the "active' zone of cartilage may differ from that of the "resting" zone

The repeating dissaccharide unit of chondroitin sulfate contains one acetamido group and one ester sulfate residue (Brimacombe and Webber 1964) Acetate and inorganic sulfate can serve as precursors to these components *via* the formation of 3'-phosphoadenosine 5' phosphosulfate (PAPS) and acetyl coenzyme A respectively (Boström and Rodén 1966) Previous studies of Boström and Månsson (1952) made it evident that much more sulfate than acetate was incorporated into sliced costal cartilage of calves This finding which was confirmed by the results of the present investigation probably reflects the fact that inorganic sulfate is the major source of PAPS (Boström and Rodén 1966) whereas free acetate is a minor contributor to the acetyl coenzyme A pool

The pool of inorganic sulfate in growing costal and epiphyseal cartilage has been estimated to be very small (Howell *et al* 1960, Greer *et al* 1968) The present results show that the various pools involved in the transfer of inorganic sulfate to chondroitin sulfate are rapidly equilibrated, as the rate of incorporation of exogenous sulfate into polysaccharide appeared to be constant from the beginning of the incubation period (Fig 2) The initial delay in the incorporation of exogenous acetate (Fig 2 Table III) may be due to the rapid consumption during the first half-hour of incubation of a large intracellular unlabeled pool, however, other explanations of this phenomenon may be equally plausible It is of interest in this context that the lag period initiating the incorporation of exogenous acetate was more pronounced in the lateral than in the medial zone thus suggesting the existence of regional differences in the mode of acetate metabolism in costal cartilage In view of these considerations labeled sulfate sems to be more suited than labeled acetate for quantitative studies of glycosaminoglycan biosynthesis

Incubation of whole cells have been extensively used in studies of glycosaminoglycan biosynthesis in various tissues The incubation period have ranged between 0 5 and 48 hrs under experimental conditions similar to those employed in the present study It should be observed that although considerable incorporation of both sulfate and acetate occurred for at least 24 hrs the incorporation rates decreased

markedly after about 2 hrs of incubation (Fig 2, Table III) Since this decrease probably reflects a loss of viability of the cartilage specimens it is evident that the results of experiments involving prolonged incubation periods should be interpreted with great care

This work was supported by grants from the Nordisk Insulinfond the Swedish Medical Research Council (B69 13X 2309), the Swedish Cancer Society (201 B68) and Gustaf V s 80 arsfond The excellent technical assistance of Miss G Backstrom and Mrs B Jansson is gratefully acknowledged

References

BELANGER L F., Autoradiographic visualization of the entry and transit of S^{35} in cartilage, bone and dentine of young rats and the effect of hyaluronidase *in vitro* *Canad J Biochem* 1954 *32* 161—169

BITTER T and H M MUIR A modified uronic acid carbazole reaction *Analyt Biochem* 1962 *4* 330—334

BOSTROM H and B MÅNSSON Exchange of the acetyl group of chondroitin sulphuric acid in slices of cartilage *Acta chem scand* 1952 *6* 1559—1561

BOSTROM H and B MÅNSSON Factors influencing the exchange of the sulphate group of the [illegible] *6* 23—37

[illegible] *The amino sugars Vol* [illegible] York 1966 45—80

[illegible] C and chondroitin In *Mucopolysaccharides* Elsevier Publishing Co Amsterdam 1964 64—91

DODGSON K. S and A G LLOYD Metabolism of acidic glycosaminoglycans (mucopolysaccharides) In *Carbohydrate metabolism and its disorders, Vol I* Ed by E Dickens P J Fandle and W J Whelan Academic Press New York 1968 169—212

GREER R B G J JANICKE and H J MANKIN Protein polysaccharide synthesis at three levels of the normal growth plate *Calcif Tis Res* 1968 *2* 157—164

HERBAI G Autoradiographic studies with ^{35}S-sulfate on somatotrophin and estrogen sensitive growth zones in rat and mouse costal cartilage *Acta physiol scand* 1970a *79* 351—358

HERBAI G Incorporation and disappearance of sulfate in different regions of mouse and rat costal cartilage *in vivo* and *in vitro* *Acta physiol scand* 1970b *79* 541—551

HOWELL D S E DELCHAMPS W REIMER and T KIEM A profile of electrolytes in the cartilaginous plate of growing ribs *J clin Invest* 1960 *39* 919—929

LINDAHL U Attempted isolation of a heparin proteoglycan from bovine liver capsule *Biochem J* 1970 *116* 27—34

MATUKAS V J and G A KRIKOS Evidence for changes in protein polysaccharide associated with the onset of calcification in cartilage *J cell Biol* 1968 *39* 43—48

MUIR H The structure and metabolism of mucopolysaccharides (glycosaminoglycans) and the problem of the mucopolysaccharidoses *Amer J Med* 1969 *47* 673—690

RADHAKRISHNAMURTHY B E R DALFERES and G S BERENSON Determination of hexosamines by gas liquid chromatography *Analyt Biochem* 1966 *17* 545—550

SCHENK R. K D SPIRO and J WIENER Cartilage resorption in the tibial epiphyseal plate of growing rats *J cell Biol* 1967 *34* 275—291

SCOTT J E Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues In *Methods of biochemical analysis Vol 8* Ed by D Glick Interscience Publishers Inc New York 1960 145—197

SILBERT J E Metabolism of polysaccharides containing glucuronic acid In *Glucuronic acid* Ed by G D Dutton Academic Press New York 1966 385—453

UMBREIT W W R H BURRIS and J F STAUFFER *Manometric techniques* Fourth ed Burgess Publishing Co Mineapolis 15 USA 1964 131—133

WEATHERELL J A and S M WEIDMANN The distribution of organically bound sulphate in bone and cartilage during calcification *Biochem J* 1963 *89* 265—267

WESSLER E Analytical and preparative separation of acidic glycosaminoglycans by electrophoresis in barium acetate *Analyt Biochem* 1968 *26* 439—444

WESSLER E Electrophoretic separation of glycosaminoglycans excreted in excess in different kinds of mucopolysaccharides In *Chemistry and molecular biology of the intercellular matrix Vol II* Ed by E A Balazs Academic Press New York 1970 895—901

WUTHIER R E A zonal analysis of inorganic and organic constituents of the epiphysis during endochondral calcification *Calcif Tiss Res* 1969 *4* [illegible]0—38

Acta physiol. scand 1970. 80 510—518

From the Department of Physiology, Karolinska Institutet Stockholm Sweden

Potentiating Action of Desmethylimipramine on the Cardiovascular Effects of Noradrenaline and Some Other Pressor Agents

By

A ÅSTRÖM

Received 8 May 1970

Abstract

ÅSTRÖM A *Potentiating action of desmethylimipramine on the cardiovascular effects of noradrenaline and some other pressor agents* Acta physiol scand 1970 80 510—518

The influence of desmethylimipramine (DMI) on the cardiovascular responses to i v injected noradrenaline (NA) adrenaline metaraminol and isoprenaline as well as to some chemically unrelated pressor agents (angiotensin vasopressin and the vasopressin analogue felypressin) was studied in anesthetized dogs and cats The cardiovascular effects of NA were potentiated very markedly by moderate doses of DMI (0 1—1 0 mg/kg) The responses to adrenaline and metaraminol were only slightly potentiated In the case of adrenaline DMI was slightly more effective in enhancing the pressor action of this amine after partial β blockade (propranolol) In analogy with the carotid occlusion response the effect on the peripheral vascular resistance elicited by electrical stimulation of the lumbar sympathetic nerves was not significantly potentiated by DMI

A method of "delayed autoperfusion was developed to record reflex vasomotor counter regulation DMI was found to reduce the activity of this counter regulation suggesting that this may be one way in which this drug potentiates the cardiovascular effects of the non-catecholamine pressor agents

Imipramine and desmethylimipramine (DMI) have been shown to possess a variety of pharmacological actions However none of these are particularly strong or specific (Domenjoz and Theobald 1959) The most striking pharmacological property of these tricyclic antidepressants seems to be their potentiation of the cardiovascular effects of injected noradrenaline (NA) as first emphasized in animal experiments by Sigg (1959 Potentiation of the pressor action of i v injected NA in patients treated with different antidepressants (e g imipramine DMI and amitriptyline) was described by Fischbach Harrer and Harrer (1966 The interaction between protriptyline and NA and adrenaline was studied in human volunteers by Svedmyr (1968) who found that the effect of NA was potentiated approximately 9 fold and that of adrenaline about 3 fold by this tricyclic antidepressant

The possible adverse effect of this drug interaction is of clinical importance e g

when NA or adrenaline are used as localizing agents to reduce the toxicity and enhance the action of local anesthetic agents In this particular case the synthetic vasopressin analogue felypressin (Octapressin®, Sandoz) seemed to be of particular interest as a localizing agent that might provide a possible alternative to the catecholamines In a study reported earlier (Åstrom 1970), no potentiation by DMI of the cardiovascular actions of felypressin was observed in the doses used in local anesthetic solutions for dental procedures (0 001—0 002 I E /kg b w)

In an extension of this previous study, larger doses of felypressin were investigated It then was observed that sometimes after DMI a slight but definite, potentiation of the cardiovascular effects of felypressin did occur This observation seemed to warrant further pharmacological investigation of the mechanisms involved in this drug interaction In the study to be reported here, the cardiovascular effects of some sympathomimetic amines (isoprenaline, metaraminol and tyramine) as well as of two other pressor agents (vasopressin and angiotensin) have been compared in anesthetized cats and dogs before and after the injection of DMI

Methods

21 cats and 9 dogs (beagles) were used in this investigation The animals were anesthetized with pentobarbital sodium (35 mg/kg) injected i p or i v (dogs)

Systemic arterial pressure was recorded from the right femoral artery and central venous pressure from the right atrium The polyethylene catheters were connected to Statham pressure transducers The heart rate meter was triggered bv the pressure peaks in the arterial pulse and recorded the intervals between beats (non linear scale) The sympathetic nerves were stimulated electrically by a square pulse stimulator (Grass Model S 4) equipped with platinum electrodes

artery Blood from the central end of the cut artery was passed through a constant flow perfusion pump which was set at a comparatively low flow rate When a drug was injected i v and elicited systemic blood pressure reactions the perfusion loop allowed the vasomotor reflexes (counter regulation) mediated by the sympathetic nerves to produce resistance changes in the perfused vascular bed which could be observed before the injected drug arrived to produce its direct effect on the vascular smooth muscles of the perfused limb In dogs as a rule

coil in order to

asomotor reaction

col was kept at

tyramine fely

(Hypertensin N

igy propranolol

Results

It was found that DMI (0 1—1 0 mg/kg) regularly potentiated the cardiovascular effects of NA both in cats and dogs (Fig 1) DMI itself (0 1—1 0 mg/kg) had a moderate pressor action of very long duration which sometimes lasted more than one hour Higher doses of DMI (2—5 mg/kg) produced a fall in blood pressure, as a rule Maximum potentiation of the pressor action of NA usually was observed

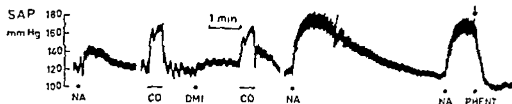


Fig 1 Dog 12 5 kg Pentobarbital anesthesia Effects on systemic arterial pressure (SAP) of i v injection of noradrenaline (NA 0 5 μg/kg) and bilateral occlusion of the carotid arteries (CO) *for 30 sec, before* and after DMI (1 mg/kg) The potentiated NA responses was effectively counteracted by phentolamine (PHENT 0 5 mg/kg)

with doses of DMI of 0 5—1 0 mg/kg Increasing the dose of DMI 2—5 times above this level did not produce any further potentiation, and high doses (10 mg/kg) usually decreased the response to NA This potentiation was readily counteracted by phentolamine as shown in Fig 1

At dose levels of 0 1—1 0 mg/kg DMI generally did not affect the response of blood pressure to carotid occlusion After higher doses of DMI a decrease in the pressor response was always observed When a slight potentiation of the carotid occlusion response occasionally was observed at dose levels of 0 1—1 0 mg/kg the response took the form shown in Fig 1 *i e* the first part of the blood pressure response was unaffected whereas as a secondary response the return to the base line pressure was delayed This pattern of slight potentiation by DMI was observed in 2 out of 13 cats and 1 out of 4 dogs It was seen more often when the carotid occlusion was prolonged (15—30 sec) After higher doses of DMI (5—10 mg/kg) the effect of carotid occlusion on blood pressure was usually decreased

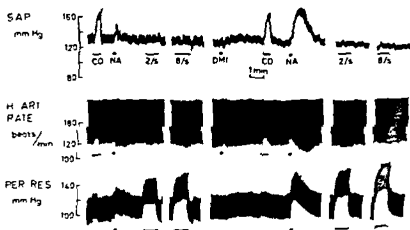


Fig 2 Dog 16 5 kg Pentobarbital anesthesia Effects of carotid occlusion (CO 15 sec) i v NA injection (0 7 μg/kg and electrical stimulation of the lumbar sympathetic nerves 4 V duration 4 msec, 2 and 8 pulses per sec) on systemic blood pressure SAP heart rate and peripheral vascular resistance in the left perfused leg (52 ml/min) before and after DMI (0 1 mg/kg)

The possible influence of DMI on peripheral sympathetic responses was studied in 3 dogs and 2 cats. The left leg was perfused at a constant rate with blood from the central end of the cannulated femoral artery. Changes in peripheral resistance were recorded as changes in the perfusion pressure. The results of such an experiment are illustrated in Fig. 2. After DMI (0.1 mg/kg), the effect of NA but not of carotid occlusion, on blood pressure and heart rate markedly potentiated. DMI only slightly enchanced the increased resistance to flow in the leg produced by electrical stimulation of the lumbar sympathetic nerves at a frequency of 2 and 8 pulses per sec. This slight potentiation of the peripheral sympathetic response is illustrated in Fig. 2 and was observed in only one additional experiment (cat). In the other three experiments that were performed in an identical manner, and included doses of DMI ranging from 0.02 to 2.0 mg/kg no increase could be detected at all. In two cases following high doses of DMI (2—5 mg/kg), there was a definite decrease in the peripheral sympathetic response to electrical stimulation.

The actions of adrenaline on blood pressure, heart rate and central venous pressure were only slightly, or not at all potentiated by the administration of DMI in doses ranging from 0.1—3.0 mg/kg. When the blood pressure effect was enhanced in animals showing a biphasic blood pressure response to adrenaline, the potentiation affected only the pressor part of the response. In an attempt to examine this effect somewhat more closely a β blocker was used to change the usual type of biphasic adrenaline effect to a more pure pressor response before DMI was administered.

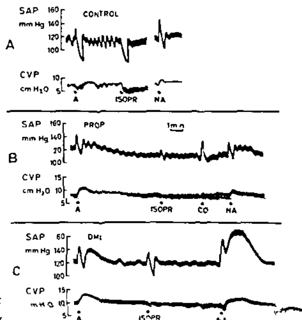


Fig. 3. Dog 12 kg. Pentobarbital anesthesia. The effects of adrenaline (A 0.3 µg/kg), isoprenaline (ISOPR 0.15 µg/kg) and NA (0.3 µg/kg) on systemic *arterial pressure* (SAP) and central venous pressure (CVP).
A at the beginning of the experiment (control).
B 3 min after propranolol 0.1 mg/kg.
C 3 min after DMI 0.5 mg/kg.
A, B and C recorded with intervals of 8 min.

The result of one of two identical experiments of this kind is illustrated in Fig 3 Propranolol 0 1 mg/kg blocked the effect of isoprenaline, and converted the biphasic adrenaline effect (Fig 3 A) into a predominantly pressor response (Fig 3 B) 10 min after DMI (0 5 mg/kg) was administered to the propranolol pretreated animal, there was a definite increase in the response to adrenaline (Fig 3 C) The usual potentiation of the action of NA on both arterial pressure and central venous pressure was seen. After DMI, isoprenaline affected systemic blood pressure in a biphasic manner (Fig 3 C)

The action of metaraminol was studied both before and after different doses of DMI A slight potentiation (10—25 %) of the response to metaraminol was observed after small doses of DMI (0 1—0 5 mg/kg) in all 5 expts in which the NA-effect was potentiated more than 100 % Similar results were obtained with nortriptyline but slightly higher doses of this tricyclic antidepressant were required to produce the same effect In 3 expts with DMI and 3 with nortriptyline it was found also that both antidepressants reduced or abolished the effect of tyramine on blood pressure

The effect of felypressin was studied over a wide dose range (0 001—0 5 I U /kg) before and after DMI (0 1—3 0 mg/kg) A slight potentiation, not more than 50 %, could be observed in 4 of the 12 expts in which the dose of felypressin was more than 0 01 I U /kg and the dose of DMI higher than 0 5 mg/kg Doses of felypressin below 0 008 I U /kg did not produce any noticeable effects on blood pressure The potentiation of the response to felypressin was never more than 50 % even if in the same experiment the effect of NA was potentiated 5—10 times

Because of the unexpected finding that DMI potentiated the pressor action of felypressin to a small but definite extent, it seemed relevant to study some other non-catecholamine pressor agents Vasopressin 0 04—0 06 I U /kg injected before and after DMI (1 mg/kg) was found to be definitely potentiated this potentiation by DMI involving predominantly the duration of the pressor action The same results were obtained in 2 dogs and 2 cats The pressor effect of angiotensin 0 01—

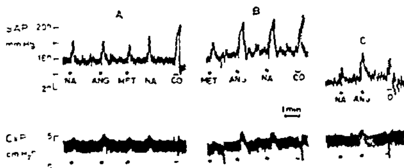


Fig 4 Cat 3.8 kg Pentobarbital anesthesia The effect of noradrenaline NA 1 μg angiotensin (ANG 0 1 μg metaraminol MET 5 μg) and bilateral carotid occlusion CO 15 sec)
A before (control
B after DMI, 0 8 mg kg
C. after phentolamine 0 5 mg/kg

0 05 μg/kg also was potentiated by DMI pre treatment (0 4—1 0 mg/kg) In a series of 4 cats the pressor effect of angiotensin was potentiated 1 2—4 times while that of NA was potentiated 2—5 times (comparison on the basis of the area under the blood pressure curves) The effect of angiotensin (0 03 μg/kg) before and after DMI is shown in Fig 4 A and B For comparison the effects of injected NA and metaraminol as well as tests with bilateral carotid occlusion were also studied in this preparation After DMI (0 8 mg/kg) the effect of metaraminol (1 2 μg/kg) was potentiated somewhat but to a much smaller degree than the action of NA or angiotensin In Fig 4 C recorded 65 min after the experiment shown in Fig 4 B and 8 min after the i v injection of phentolamine in fractionated doses (total dose 0 5 mg/kg) had produced a partial *a* blockade the pressor effect of NA was de pressed below the control value but the effect of angiotensin remained potentiated

In most of the experiments in this investigation the changes in peripheral vascular resistance were recorded by a delayed autoperfusion method (see Methods) Often it was observed that after a dose of DMI sufficient to produce a potentiation of the response to NA the elicited reflex counter regulation was reduced in size This effect is illustrated in Fig 5 Fig 5 A shows the effect of NA on heart rate systemic arterial pressure central venous pressure and peripheral vascular resistance (delayed autoperfusion) In A 2 5 and 1 0 μg NA produced a dose dependant response in all parameters recorded Active counterregulation was indi cated by the decrease in peripheral vascular resistance that occurred while the systemic arterial pressure was elevated and before the injected NA had reached the perfused vascular bed In 5 B recorded 10 min after DMI (1 mg/kg) 1 μg NA produced about the same effect on arterial blood pressure as 2 5 μg NA had pro

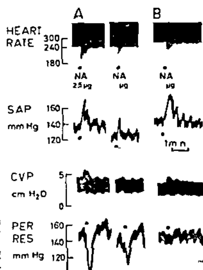


Fig 5 Cat 2 2 kg pentobarbital anesthesia The effect of 2 5 μg and 1 μg noradrenaline (NA) on heart rate systemic arterial pressure (SAP) central venous pressure (CVP cm H_2O) and peripheral vascular resistance (PER RES delayed autoperfusion) before and for 1 μg NA after the injection of DMI 1 mg/kg) — *Note* no reflex fall in peripheral resistance was elicited after DMI

duced before DMI The effects on heart rate and central venous pressure of 1 µg NA after DMI were also about the same as those produced by 2 5 µg NA prior to DMI administration *However, in the tracing of peripheral vascular resistance there was no sign of counter regulation mediated by the nervous system*

Discussion

The results regarding the potentiation of NA after various doses of DMI are in general agreement with those reported previously, *e g* Osborne and Sigg (1960) The findings also support the conclusion drawn by Kaumann *et al* (1965) that in sharp contrast with NA, the effects of adrenaline and isoprenaline on blood pressure heart rate and cardiac contractile force are not significantly modified by DMI

Since it was recognized that an uptake mechanism is probably the most important means by which the action of free catecholamines is terminated (Axelrod *et al* 1959) a great many analyses have been made of the mechanisms of catecholamine uptake by sympathetic nerves and nerve granules as well as catecholamine granules from the adrenal medulla (*e g* Carlsson Hillarp and Waldeck 1963 Stjarne 1964 Malmfors 1965 Euler 1966) It was found that cocaine and imipramine (and DMI) blocked the uptake mechanisms in several systems (Hertting *et al* 1961 Dengler *et al* 1962 Hillarp and Malmfors 1964)

Although uptake mechanisms associated with the membrane of sympathetic nerves and the intraneuronal granules have been emphasized lately a less specific type of uptake coupled with the enzymatic inactivation of catecholamines in the liver and other organs (Euler 1956) may be expected to be of great importance when amines are injected i v as in the experiments described here Ginn and Vane (1968) studied the uptake of adrenaline NA and isoprenaline in the peripheral circulation and concluded that an uptake other than that by sympathetic nerves must contribute greatly to the physiological disposition of circulating catecholamines Sulser and Sokoro (1965) pointed out that whereas NA uptake is blocked by DMI both *in vitro* and *in vivo* potentiation of endogenous and exogenous NA cannot be demonstrated in *in vitro* preparations *e g* aortic strips rat auricle and rabbit ileum

In this investigation it was found that DMI potentiates the action of NA much more than that of adrenaline whereas the action of isoprenaline is unaffected This indicates that DMI blocks an uptake pool which has a high affinity for NA a lower affinity for adrenaline and practically no affinity for isoprenaline This order of affinity for the uptake mechanisms is the same as that which has been described previously for these amines using different techniques (Whitby *et al* 1961 Anden *et al* 1964 Ross and Renyi 1966) In the case of isoprenaline the axon membrane seems to constitute a barrier to the passage of the amine since isolated nerve granlues take up isoprenaline to the same extent as NA and adrenaline (Euler and Lishajko 1967)

A blockade of the peripheral sympathetic nervous tissue uptake of catecholamines would seem unlikely as the sole explanation for the potentiating action of DMI

which was observed in this investigation The difference between the potentiation of NA and adrenaline was greater than expected on the basis of a difference in affinity to the uptake mechanisms in the sympathetic nerves Also, at maximum potentiation of injected NA, there were no conclusive signs of a potentation of the sympathetic nerve responses (carotid occlusion and electrical stimulation of the sympathetic nerves, Fig 2)

The finding that the action of metaraminol was enhanced only slightly after DMI deserves some comment Metaraminol is taken up most readily by the sympathetic nerves, and this uptake is most effectively blocked by DMI (Carlsson and Waldeck 1965) Metaraminol is considered to be a sympathomimetic amine with both a direct and indirect mode of action A blockade of uptake by DMI could therefore have been expected to increase the concentration of the amine at the receptor site However, since at the same time less NA would be released, the net result could be expected to reflect an approximate balance of these opposing effects

The results presented here show that DMI pretreatment also definitely increases the pressor effect of agents like vasopressin, its analogue felypressin, and angiotensin The potentiation of vasopressin and angiotensin was usually greater than that of adrenaline, but was always less than the maximum potentiation of NA As with the potentiation of the action of NA, a maximum effect was obtained with moderate doses (0 1—1 0 mg/kg) It is not known if DMI decreases the binding or uptake of these pressor agents Another possible explanation for this potentation of the pressor effect of vasopressin and angiotensin is suggested by the results of the 'delayed autoperfusion" experiments In these experiments the recordings of peripheral arterial resistance showed that, after treatment with DMI, elevations of systemic arterial pressure was not accompanied by a fall in peripheral resistance indicating that vasomotor counter-regulation was reduced

It should be added that the collateral circulation to the perfused limb was not completely interrupted, in order to limit the trauma of the operative procedure Therefore, with the method used, an increase in systematic arterial blood pressure (particularly in dogs) tended to mask a fall in perfusion pressure because of retrograde transmission of pressure via the collateral vessels Because of this care was taken to compare the reflex counter-regulation elicited by equipressor doses of NA both before and after DMI as in Fig 5 A decreased effectiveness of vasomotor counter regulation would also seem to explain the finding that the action of adrenaline could be more markedly enhanced by DMI after partial β blockade (Fig 3) It should be added that when arterial blood pressure was lowered with acetylcholine, counter regulation (peripheral vasoconstriction) in these preparations was unimpaired It is proposed therefore that the decrease in counter-regulation which followed the i v injection of the pressor agents was due to the fact that the injected DMI had elevated arterial blood pressure (Fig 1) and had already decreased the sympathetic efferent discharge to such a low value that further "turning off" of prevailing vasomotor tone was not possible The increase in systemic arterial pressure after moderate doses of DMI may result from a potentiation of small amounts of

circulating NA and, possibly, from some stimulating action of DMI on the heart (Kaumann *et al* 1965)

This investigation was supported by a grant from "Therese och Johan Anderssons Minne The skilful technical assistance of Mrs Annika Rosen is gratefully acknowledged

References

ANDEN N E H CORRODI M ETTLES E GUSTAFSSON and H PERSSON, Selective uptake of some catecholamines by the isolated heart and its inhibition by cocaine and phenoxybenzamine *Acta pharmacol* (Kbh) 1964 *21* 247—259

ÅSTROM A *Toxic effects of local anaesthetic agents* Proc IV World Congr Anaest (London Sept 1968) Progress in Anaesthesiology 293—296 Published in Exerpta Medica Foundation 1970

AXELROD J H WEIL MALHERBE and R TOMCHICK The physiological disposition of H[3] epinephrine and its metabolite metanephrine *J Pharmacol exp Ther* 1959 *127* 251—256

CARLSSON A N A HILLARP and B WALDECK Analysis of the Mg ATP dependent storage mechanisms in the amine granules of the adrenal medulla *Acta physiol scand* 1963 *59* Suppl 215 1—38

CARLSSON A and B WALDECK Mechanism of amine transport in the cell membranes of the *adrenergic nerves Acta pharmacol (Kbh) 1965 22 293—300*

DENGLER H J I A MICHAELSON H E SPIEGEL and E TITUS The uptake of labelled norepinephrine by isolated brain and other tissues of the cat *Int J Neuropharmacol* 1962 *1* 23—38

DOMENJOZ R and W THEOBALD Zur Pharmakologie des Tofranil® (N (3-dimethylaminopropyl) iminodibenzyl hydrochlorid) *Arch int Pharmacodyn* 1959 *120* 450—489

EULER U S v *Noradrenaline chemistry physiology pharmacology and clinical aspects* Charles C Thomas Publisher Springfield Ill U S A 1956

EULER U S v Release and uptake of noradrenaline in adrenergic nerve granules *Acta physiol scand* 1966 *67* 430—440

EULER U S v and F LISHAJKO The uptake of isoprenaline in nerve granules *Int J Neuropharmacol* 1967 *6* 431—434

FISCHBACH R G HARRER and H HARRER Verstarkung der Noradrenaline Wirkung durch Psychopharmaka beim Menschen *Arzneimittel Forsch* 1966 *16* 263— 265

GINN R and J R VANE Disappearance of catecholamines from the circulation *Nature* (Lond 1968 *219* 740—742

HERTTING G J AXELROD and L G WHITBY Effect of drugs on the uptake and metabolism of [3]H norepinephrine *J Pharmacol exp Ther* 1961 *134* 146—153

HILLARP N A and T MALMFORS Reserpine and cocaine blocking of the uptake and storage mechanisms in adrenergic nerves *Life Sci* 1964 *3* 703—708

KAUMANN A N BASSO and P ARAMENDIA The cardiovascular effects of N () methylaminopropyl imino-dibenzyl) HCl (Desmethylimipramine and guanethidine *J Pharmacol exp Ther* 1965 *147* 54—64

MALMFORS T Studies on adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 *64* Suppl 248 1—93

OSBORNE M and E B SIGG Effects of imipramine on the peripheral autonomic system *Arch int Pharmacodyn* 1960 *129* 273—289

ROSS S B and A L RENYI Uptake of some tritiated sympathomimetic amines by mouse brain cortex slices in vitro *Acta pharmacol* (Kbh) 1966 *24* 297—309

SIGG E B Pharmacological studies with Tofranil *Canad psychiat Ass J* 1959 *4* 575—5?3

STJARNE L Studies of catecholamine uptake storage and release mechanisms *Acta physiol scand* 1964 *62* Suppl *228*

SULSER F and F SOROKO On the role of rate of brain norepinephrine release in the anti [illegible] (Berl) 1965 *8* 191—200

SV[illegible] of the circulatory effects [illegible]

W[illegible] orepinephrine in animals [illegible]

J Pharmacol exp [illegible]

Acta physiol. scand 1970. 80. 519—527

From the Department of Pharmacology, Karolinska Institutet Stockholm, Sweden

Studies on the Mechanism of Action of Lithium Ions I. The Effect of Lithium Ions on the Impulse Activity of the Crayfish Stretch Receptor Neuron

By

E Giacobini and M Stepita Klalco

Received 12 May 1970

Abstract

Giacobini, E and M Stepita Klalco *Studies on the mechanism of action of lithium ions on the impulse activity of the crayfish stretch receptor neuron* Acta physiol. scand 1970 *80* 519—527

When the extracellular Na^+ was replaced by lithium (Li^+) the frequency of the impulses elicited by stretching the stretch receptor of crayfish was changed and then inhibited This effect was not due to the lack of extracellular Na^+ Higher firing rate facilitated and low extracellular K^+ delayed the Li^+ effect. Ouabain alone (10^{-4} M) changed the impulse activity in a similar manner to Li^+ replacement Ouabain (10^{-5} M) decreased the rate of recovery during wash-out after Li^+ replacement. The Li^+ induced increase in the frequency of the impulse activity was probably the result of a lower intracellular K^+ concentration, the block of the spike activity being due to the accumulation of Li^+ in the cell The Na pump participated in the recovery of the spike activity after Li^+ replacement

---

It has been known for many years that lithium (Li^+) can almost completely replace Na^+, in the mechanism of the action potential in nerve fibers (Overton 1902 Hodgkin and Katz 1949), in skeletal muscle of frog (Keynes and Swan 1959) and in non-myelinated fibers of mammals (Ritchie and Straub 1957) The extracellular replacement of Li^+ for Na^+ results in a depolarization of the cell membrane of the crayfish stretch receptor neuron (Obara and Grundfest 1968) as well as in other cells These investigations supported the hypothesis that Li^+ induced depolarization by interferring with the Na pump The present study was undertaken to examine such a possibility

Material and Methods

The stretch receptor neuron (SRN) from the fresh water crayfish (*Astacus fluviatilis*) was used The receptor from the second and third abdominal segment with a 2—3 mm long segment of nerve containg the axon was dissected out Propagated spike activity was recorded extracellularly from the nerve using a recording electrode similar to that described by Easton

(1960). After dissection, the cell was left in the physiological solution for at least 3 hrs. After this period impulse activity elicited by maintained stretch became stable, as did the ionic content of the SRN (Giacobini *et al.* 1967). The solution in the perfused chamber was stirred continuously. The perfusion rate exchanged 95 % of the volume of the chamber in 1 min and was constant throughout the experiment.

A modified van Harreveld solution (1936) was used throughout the measurements (mM): NaCl 207, KCl, 5.4, $CaCl_2$, 18.0, $MgCl_2$, 2.5, $KHCO_3$, 2.4, glucose, 11.1. The pH was maintained between 7.4–7.5 and the temperature between 17–19° C. In the Li^+ containing media a corresponding part of NaCl (3 %–100 %) was stoichiometrically replaced by LiCl. Unless otherwise stated, Li^+ replacement refers to 100 % replacement of the extracellular Na^+ by Li^+. In the low K^+ medium (2.4 mEq) the 5.4 mM KCl was omitted.

Results

The effect of Na^+ replacement by Li^+

The extracellular replacement of Na^+ by Li^+ (25 %–100 %) caused an initial small transient decrease in the frequency of the impulse activity elicited by maintained stretch, which was immediately followed by an increase, and then an abrupt fall to zero (Fig. 1). Replacement of less than 25 % however, induced only a decrease in the frequency. After washing with normal physiological solution the previous activity was restored.

The time between the Li^+ replacement and the maximum spike activity depends on the frequency of firing before replacement. The higher the initial frequency the steeper the increase of the frequency during replacement (Table I).

The time during which spike activity was maintained, that is the interval between the beginning of Li^+ replacement and the stop of spike activity, was used to measure the Li^+ effect. When this interval is plotted against the concentration of Li^+ in the medium it yields a curve which is a simple function of the concentration (Fig. 2). During wash-out the impulse activity was restored, then decreased and after reaching a minimal level the frequency increased until it stabilized (Fig. 1).

TABLE I. Influence of the frequency of the spike activity on the effect of Li^+. In this case the normal medium was not exchanged in 1 min but rather slowly over a 10 min period. This was done to slow down the changes of the frequency during Li^+ replacement. The frequency indicated is the stable frequency before Li^+ replacement. Time to peak activity refers to the interval from the beginning of the Li^+ replacement to the moment of the highest firing frequency attained.

Cell no.	Initial frequency imp/min	Time to peak activity (min)	Peak activity imp/min
1	425	19	1 950
	125	32	800
2	430	15	1 700
	125	33	990
3	350	11	1 280
	250	14	960
4	405	12	1 020
	175	24	680
5	400	14	2 400
	150	23	770

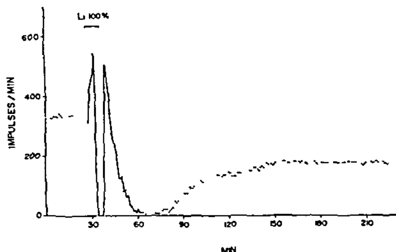


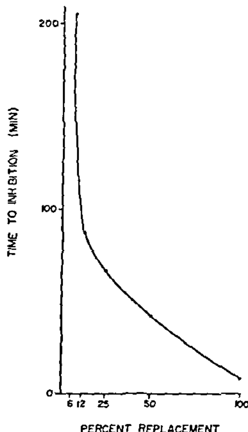
Fig 1 The effect of Na^+ replacement by Li^+ (100 %) The interval of replacement is indicated by the bar

The frequencies measured during the wash-out, when plotted in a semilogarithmic diagram (the frequency in log scale) as a function of time, can be approximated by straight lines for both the decreasing and the initial part of the increasing phases of the curve (Fig 5) *Their corresponding time constants (the time interval to reach* 63 % decrease or 2 7 times increase in the frequency) were $T_1 = 9\ 2 \pm 2\ 8$ min (n = 12) for the decreasing and $T_2 = 18\ 2 \pm 3\ 4$ min (n = 7) for the increasing phase If the initial firing frequency was not high enough (<350—400/min) the frequency dropped to zero during wash-out However the activity could be elicited again by stretching The frequency slopes during wash out after Li replacement at high concentrations (25 %—100 %) are the same At lower Li concentrations (3 % 6 % 12 %) only a continuous increase of frequency during recovery was seen

The level of the stable frequency after complete recovery was always lower than before Li replacement (Fig 1) This difference could not be accounted for by a spontaneous slow decrease of activity

The effect of K

The effect of Li was dependent upon the extracellular concentration of K With complete replacement of Na by Li in the presence of a decreased concentration of K (2 4 mEq) there was a substantial increase in the duration of the repetitive activity (time of inhibition = $20\ 1 \pm 3\ 4$ min, n = 7) (Fig 3) The time constants for the frequency changes during Li wash-out in low K were not different from the controls There was no further significant increase in the duration of the repetitive activity using a K free medium however, there was no recovery after Li wash



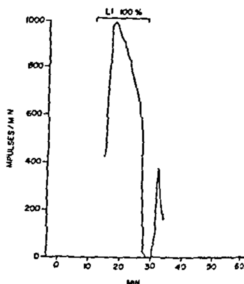


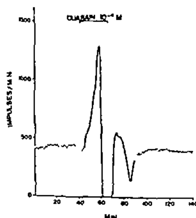
Fig. 2. Li dose—response curve. For Li concentrations corresponding to 25, 50, 100 % of Na in the normal solution (207.7 mEq) the resulting inhibition was complete. Using lower concentrations despite the blockade of the activity the repetitive firing could be elicited by an increased stretch. Time in min. The initial firing rate was adjusted between 100—200/min.

Fig. 3. The effect of complete Na replacement by Li in low K (2.4 mEq) medium. Note the prolonged interval between the increase of the frequency and the block of the activity as compared to Fig. 1.

The effect of Na replacement by choline

In order to decide whether the effect of Li was due to the lack of extracellular Na during Li replacement or not, choline was substituted for Na. Before the addition of the choline medium, the cell was atropinized (10 mg atropine sulphate/liter) for half an hour in order to prevent choline blockage of activity.

Total replacement by choline induced an instantaneous increase of activity which then decreased and ceased after approximately 5 min. The preparation of the crayfish stretch receptor neuron contains a layer of glia covering the cell body and axon. This may act as an extracellular compartment of sodium which is able to maintain the firing. There were no slow changes of the activity during choline wash-out. The stable frequency after wash-out is higher than the initial frequency before treatment.



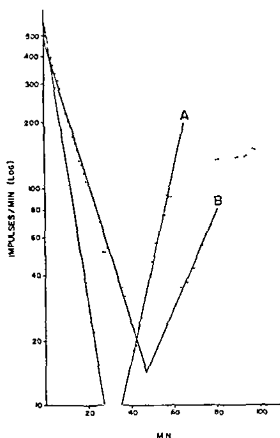


Fig 4 The effect of ouabain (10^{-4}) The duration of the treatment is indicated by the bar

Fig 5 Changes in frequency during wash out after complete Li replacement (100 %) in the medium without (A) and with (B) ouabain (10^{-5} M)

The effect of ouabain

Ouabain alone (10^{-4} M) had an effect similar to that of Li (Fig 4) The frequency increased before the block was restored by wash out with physiological solution decreased and finally increased before reaching a stable level The duration of the spike activity was longer than after Li replacement The time constant of the decreasing and increasing phases (1 5—2 5 min) were equal and are substantially shorter than the time constants during Li wash out (9 2 18 2 min respectively) The other main difference between the effect of ouabain and Li were 1) absence of the initial decrease in the frequency 2) a non-exponential change of the frequency of activity during the first minutes of wash-out and 3) the stable frequencies before and after ouabain were equal

The effect of ouabain and Li

A low concentration of ouabain (10 M) alone produced no change in the frequency of firing Qualitatively, the total replacement of extracellular Na by Li in the presence or absence of 10 ⁻ M ouabain led to the same changes in frequency Quantitatively, there was a difference namely the prolongation of both frequency time constants during Li wash-out in the presence of ouabain ($T_1 = 25.8 \pm 4.2$ $T_2 = 43.7 \pm 5.7$ n = 5) (Fig 5)

Discussion

Lithium stops the generation of the impulse activity of the stretch receptor neuron in the axonal hillock region The axon of such a silent neuron is still able to generate a propagated action potential when stimulated electrically (Obara and Grundfest 1968) The measured changes in the frequency correspond to the polarization of the hillock region which is mostly influenced from the side of the somatic membrane since the depolarization of the axonal membrane is much less pronounced (Obara and Grundfest 1968)

Lithium prior to its stop of the impulse activity of the SRN was found to cause an initial decrease in the frequency followed by an increase These corresponded to a hyperpolarization and then a depolarization of the soma membrane (Obara and Grundfest 1968) A decrease in the frequency was measured over the entire range of the Li replacement from 3 % to 100 % This was followed by an increase in the frequency only when Li replacement was greater than 25 % During 12 % replacement there was only decrease in the frequency During 25 % replacement the decrease was followed by an increase It is reasonable to expect that it is between these two values (concentrations of Li) where the effect of Li which increases the frequency (the depolarization) begins

The decrease of the frequency which occurred immediately upon the replacement with Li above 25 % may have corresponded to the initial hyperpolarization which has been observed by many workers (Lundberg 1951 Huxley and Stampfli 1951 Gallego and Lorente de No 1952 Cerf 1955 Yonemura and Sato 1967) This effect has been explained by a lower resting permeability for Li as compared with Na ($P_{Li}/P_{Na} = 0.6$–0.7) (Huxley and Stampfli 1951 Armett and Ritchie 1963 Yonemura and Sato 1967) Obara and Grundfest (1968) however did not describe such hyperpolarization in SRN

Above 25 % Li replacement the transient decrease of the frequency was followed by an increase reflecting the depolarization of the soma membrane Depolarization during Li replacement has been observed in several kinds of cells Gallego and Lorente de No 1952 Cerf 1955 Ritchie and Straub 1957 Bohm and Straub 1962 Yonemura and Sato 1967 Obara and Grundfest 1968 Rose and Loewenstein 1969) The depolarization induced by Li in the SRN was explained as being due to inhibition at the electrogenic Na pump (Obara and Grundfest 1968 This is an

acceptable explanation in frog skeletal muscle cells whose intracellular K content does not decrease (Yonemura and Sato 1967) In the SRN however the decreased intracellular K coincides with the depolarization (Stepita *et al* 1969 Giacobini *et al* 1970) The pumping rate of K into the cell is dependent on intracellular Na (Skou 1965) and after Li replacement the intracellular Na decreased (Yonemura and Sato 1967 Giacobini *et al* 1970) Li replacement thus decreases the pumping rate of K resulting in a decreased intracellular K which is the main reason for depolarization This did not rule out a depolarization of different nature which might occur prior to the decrease in intracellular K Our results showed that the time course for the effect of Li on the spike activity could be modified by both the extracellular K concentration and the initial impulse activity In the case of low extracellular K there was an increase in the duration of repetitive activity Under these conditions the intracellular K concentration was decreased and the intracellular Li was lower than when Li was replaced in a normal K medium (Giacobini *et al* 1970) The lower Li content in cells with longer firing showed that the block depended on the intracellular Li concentration The observed action of the impulse activity in speeding up the effect of Li supports similar findings in mammalian sympathetic ganglia (Pappano and Volle 1967) and may possibly be interpreted as an increased influx of Li

Ouabain alone (10^{-4} M) produced an effect similar to that of Li Ouabain induces both a build up in the intracellular Na and a decrease in intracellular K in the SRN (Giacobini *et al* 1967) Extracellular replacement of Na by Li results in an intracellular build up of Li (Yonemura and Sato 1967) and a decrease in intracellular K in the SRN (Giacobini *et al* 1970) Since Na and Li have been demonstrated to behave identically with regard to the generation of the action potential (Moore *et al* 1966) ouabain treatment should result in similar changes in the spike activity as extracellular replacement of Na by Li

Obara and Grundfest (1968 have shown that Li blocks the spike activity at a lower level of depolarization than is required for the block induced by an injected current. Ouabain alone (10^{-4} M) results in a longer duration of the spike activity than is seen with 100 % Li replacement and ouabain induces a continuous increase of intracellular Na (Giacobini *et al* 1967) This situation resembles a decrease in the concentration gradient of an ion generating the action potential (Obara and Grundfest 1968) From this point of view the slower effect of ouabain as compared to Li might mean that after completely blocking the ouabain sensitive component of the Na pump the rate of influx of Na is lower than that of Li

The changes in the frequency during Li wash-out after Li replacement reflect the recovery processes taking place in the intracellular distribution of ions The biphasic changes in the frequency corresponded to the dual effect of Li (>25 %) replacement This assumption is based on the fact that the biphasic changes were absent in cells showing only a decrease in the frequency during Li (<25 %) replacement If the curve during wash out had corresponded to the decrease of intracellular Li and Na after Li alone or ouabain alone the efflux of Li w[illegible]

slower than the efflux of Na, since this was the ratio between the time constants

The slow recovery after Li replacement in ouabain (10^{-5}) relates the changes during the recovery process with the Na pump Whether an active efflux of L^+ ions is present or the observed changes simply reflect the result of the transport of other ions remain to be established

Our results can be summarized as follows

1 The effect of Li was accentuated by the impulse activity
2 Low extracellular K (2 4 mM) increased the time for which the cell was able to generate the repetitive activity during Li replacement
3 *Ouabain alone (10^{-4} M)* changed the impulse activity in a similar way to Li replacement
4 Ouabain (10^{-5} M) medium increased the frequency time constants during wash out after Li replacement

In conclusion it has been shown that Li alters and finally inhibits the impulse activity of the SRN These alterations are specific for Li and are not due to the lack of extracellular Na The Li induced increase in the frequency of firing is probably the result of a lower intracellular K, the complete inhibition of the spike activity is probably due to the accumulation of Li in the cell The Na pump participates in the recovery of the spike activity after Li replacement

This work was supported by grant NB 04561 06 from the U S Public Health Service and the Swedish Medical Research Council projects nr B70 14X 246 06 B69 14X 2688 01G K69 14X 2688 G01K and B70 14X 2688 02 to E G

We are indebted to Dr H Hillman Dept of Biological Sciences University of Surrey Guildford Surrey England for advice and criticism of the manuscript

References

Armett C J and L M Ritchie On the permeability of mammalian non myelinated fibres to sodium and to lithium ions *J Physiol* (Lond) 1963 *165* 130—140

Bohm H W and R W Straub Der Effekt von Lithium Ionen auf das Ruhepotential von markhaltigen Nervenfasen *Pflugers Arch ges Physiol* 1962 *274* 468—479

Carmeliet E E Influence of lithium ions on the transmembrane potential and cation content of cardiac cells *J gen Physiol* 1964 *47* 501—530

[illegible] le nerf de

[illegible] 1313

[illegible] upon frog

[illegible] n isolated

nerve cells *Acta physiol scand* 1967 *71* 391—400

Giacobini E S Hovmark and M Stepita Klauco Studies of the mechanism of action of lithium (Li) II Potassium sensitive influx of lithium ions into the crayfish stretch receptor neuron as determined by microflamephotometry *Acta physiol scand* 1970 *80* 528—532

Hodgkin A L and B Katz The effect of sodium ions on the electrical activity of the giant axon of the squid *J Physiol* (Lond) 1949 *108* 33 77

Huxley A F and R Stampfli Effect of potassium and sodium on resting and action potentials of single myelinated fibres *J Physiol* (Lond) 1951 *112* 496—508

Keynes R D and R C Swan The permeability of frog muscle fibers to lithium ions *J Physiol* (Lond) 1959 *147* 626—638

Lundberg A On the effect of temperature on the depolarization of frog nerve fibers *Acta physiol scand* 1951 *22* 348—364

MOORE J W N ANDERSON M BLAUSTEIN M TAKATA J Y LETTVIN W F PICKARD, T BERNSTEIN and V POOLER, Alkali cation selectivity of squid axon membrane *Ann N Y Acad Sci* 1966 *137* 818—829

OBARA S and H GRUNDFEST Effect of lithium on different membrane components of cray[illegible]

O[illegible]

PA[illegible]

RITCHIE, J M and E W STRAUB The hyperpolarization which follows activity in mammalian non medullated fibres *J Physiol* (Lond) 1957 *136* 80—97

ROSE B and W R LOEWENSTEIN Depression of junctional membrane permeability by substitution of lithium for extracellular sodium. *Biochim biophys Acta* (Amst) 1969 *173* 146—148

SKOU J C Enzymatic basis for active transport of Na and K across cell membrane *Physiol Rev* 1965 *45* 596—617

STEPITA KLAUCO M E GIACOBINI and S HOVMARK *Mechanism of action of Lithium studied on an isolated stretch receptor neuron preparation (SRN)* II Int Meeting Int Soc Neurochem (Milano 1969) Ed by R Paoletti R Fumagalli and S Galli Tamburi Ed Milano 1969 p 379

VAN HARREVELD A, A physiological solution for fresh water crustaceans *Proc Soc exp Biol* (N Y) 1936 *34* 428—432

YONEMURA K and M SATO The resting membrane potential and cation movement in frog muscle fibers after exposure to lithium ions *Jap J Physiol* 1967 *176* 678—697

Acta physiol. scand. 1970. 80. 528—532
From the Department of Pharmacology, Karolinska Institutet, Stockholm Sweden

Studies on the Mechanism of Action of Lithium Ions II. Potassium Sensitive Influx of Lithium Ions into the Crayfish Stretch Receptor Neuron Determined by Microflamephotometry

By

E GIACOBINI, S HOVMARK and M STEPITA-KLAUCO

Received 12 May 1970

Abstract

GIACOBINI E S HOVMARK and M STEPITA-KLAUCO *Studies on the mechanism of action of lithium ions II Potassium sensitive influx of lithium ions into the crayfish stretch receptor neuron determined by microflamephotometry* Acta physiol scand 1970 80 528—532

The complete replacement of the extracellular Na by lithium (Li) (in 7 8 mM K medium) leads to an accumulation of Li and a simultaneous decrease in Na and K in the stretch receptor neuron Li replacement in a medium with a lower K concentration (2 4 mM) decreases the quantities of both K and Li The possibility of a K sensitive carrier transporting Li into the cell is suggested

In a previous investigation it was found that the ability of the stretch receptor neuron (SRN) to generate spikes during complete replacement of Na by lithium (Li) was substantially prolonged in the presence of low extracellular K (2 4 mM) (Giacobini and Stepita-Klauco 1970)

Obara and Grundfest (1968) have shown that Li blocks the spike activity by a mechanism which cannot be accounted for completely by depolarization Thus the hyperpolarization of the neuronal membrane which occurs in the low K medium is not a sufficient explanation for the delay in the Li blockade of the spike activity It seems probable that with low extracellular K there may be an alteration in the intracellular concentrations of ions during Li replacement In order to clarify this point it was decided to estimate the changes in Na K and Li in the SRN preparation The preparations were left in a medium in which Na was replaced by Li for a period long enough to stop their firing in the medium with a normal K concentration but not long enough to stop the spike activity in the low K medium

Material and Methods

a) The following incubating media were used,

1 *Normal medium* (modified van Harreveld solution, 1936)
mM NaCl 207 7, KCl 5 4, $CaCl_2$ 18 0, $MgCl_2$ 2 5, $KHCO_3$ 2 4 glucose 11 1

2 *Low K⁺ medium* As solution 1 but with the KCl omitted

3 *Rinsing solution* Choline chloride 246 2 mM

The deionized double distilled water used for solutions had a conductivity less than 10^{-7} Siemens the pH of all solutions was between 7 4—7 5 and the temperature was maintained between 17° and 19° during the experiments During Li^+ replacement the NaCl in solutions 1 or 2 was replaced by LiCl stoichiometrically

b) *Preparation*

The stretch receptor neuron from fresh-water crayfish (*Astacus fluviatilis*) was dissected as previously described (Giacobini and Stepita Klauco 1970) It was then transferred to a small Petri dish containing 2 ml of normal or low K^+ medium The preparation was left without stretching in this medium for at least 3 hrs, which is the time necessary for stabilizing the ionic content and the impulse activity (Giacobini *et al* 1967, Giacobini and Stepita Klauco

volume of the preparation The trimming definitely makes a large contribution to the experimental errors in this study The listed values of cations (Table I) reflect the scatter of our data

TABLE I Na^+, K^+ and Li^+ in the SRN before and after the replacement of extracellular Na^+ by Li^+. (Mean ± S D)

	K^+ in medium (mM)	Na^+ (mol 10^{-12})	K^+ (mol 10^{-12})	Li^+ (mol 10^{-12})
Controls	7 8	175 ± 75 (9)	460 ± 45 (9)	—
	2 4	215 ± 45 (6)	455 ± 45 (5)	—
Treated	7 8	17 ± 8 5 (15)	380 ± 75 (11)	295 ± 50 (8)
	2 4	22 ± 9 0 (10)	245 ± 90 (8)	196 ± 45 (7)

c) *Measurement of ions*

Before the analysis the SRN was transferred rapidly by means of a stainless steel wire (Kanthal) of 15 μ diameter from the last incubating medium to the rinsing solution It was rinsed for 1 min in choline chloride solution and then picked up on the tip of a 50 μ thick wire (80 % Pt 20 % Ir) and dried in air for 3 min The wire was placed on the sample carrier of the integrating flamephotometer for estimation of the Na K and Li contents The integrating flamephotometer described by Carlsson *et al* (1966 1967) was used Interference filters for the light separation had the following specifications (Schott Gen Mainz) Na maximal transmittance 60 % at 586 nm and a half intensity bandwidth 18 nm K maximal transmittance 39 % at 764 nm, half intensity bandwidth 15 nm Li maximal transmittance 42 % at 672 nm, half intensity bandwidth 12 nm The instrument was calibrated by repeated analysis of similar quantities of standard solutions containing KCl LiCl and NaCl in the proportions 10 2 5 1

Results

The quantity of Na and K in the stretch receptor neuron was not significantly influenced by lowering the medium K concentration from 7 8 to 2 4 mM (Table I) After 10 min of total Li replacement in normal or low K medium, the content of

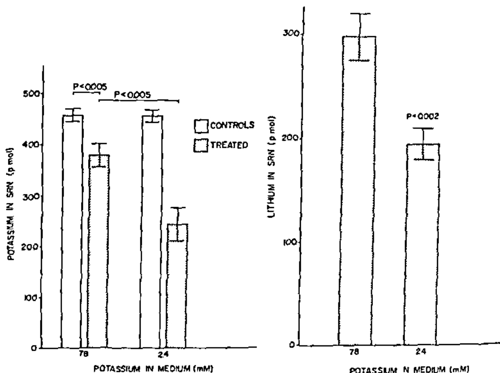


Fig 1 Variations in the K content of the SRN as a result of Li replacement The extracellular *Na was replaced by Li for 10 min in media containing 2 4 or 7 8 mM K (Mean ± S E)*

Fig 2 Lithium ion in the SRN after 10 min of extracellular Li replacement in media containing 2 4 or 7 8 mM K (Mean ± S E)

both Na and K decreased in the SRN After Li replacement the decrease of Na was not significantly different between media of 2 4 or 7 8 mM K However the K content of the SRN was significantly lower in the medium containing 2 4 mM K as compared to normal medium (7 8 mM Fig 1)

The quantity of Li in the SRN after 10 min of complete medium Li replacement depended on the extracellular K concentration In the medium with 2 4 mM K, Li was significantly lower in the SRN than after extracellular Li replacement in the medium with 7 8 mM K (Fig 2)

Discussion

The extracellular replacement of Na by Li led to an accumulation of Li in the cell and a decrease in both Na and K in the SRN Our observations in the SRN are in agreement with the results obtained in frog muscle by Yonemura and Sato (1967) However there was no change in the intracellular K^+ content in their preparation The decrease of K in the SRN might be explained as a leakage of K from the cell and the pronounced effect in the low K medium *could be due to the*

increased outward concentration gradient The amount of Li in the stretch receptor neuron in low K medium was also decreased as compared to Li replacement in normal medium despite the fact that during Li replacement the concentration gradient for Li was in favour of the inside of the cell In such a case the Li content of the cell should have increased Since the opposite occurred it would seem that the rate of influx of Li into the cell may be controlled by the extracellular K concentration This would be possible if there was competition between these two ions for the same transport mechanism. The alternative explanation would be that in the low K medium the cell was hyperpolarized over the whole 10 min and thus the spontaneous discharge rate during this period might have been slower This would lead to a less accumulation of Li Since the stretch receptor neuron was able to fire longer than controls under these conditions (Giacobini and Stepita Klauco 1970) this explanation is unlikely

Since we at present do not have data about the other cations and anions or the water content of the tissue we cannot interpret the finding that the total cations in 24 mM K media are substantially less than in the other conditions

Potassium ions accumulate in the cell by an active transport process This mechanism is controlled by the extracellular concentration of K and the intracellular concentration of Na (Skou 1965) Decreasing the gradient of either or both of these ions decreases the pumping rate resulting in a diminished K content in the cell (Garrahan and Glynn 1967) There is a similarity between the effect of Li and that of K upon the (Na—K) activated ATPase carrier mechanism however Li affinity for this enzyme is lower than that of K and the enzyme activation is less (Skou 1965) With the Li activation of the enzyme Li occupies the K site which is normally used for the exchange of K for Na During the replacement of the extracellular Na by Li the extracellular Li was 26 times higher than the extracellular K This ratio favours the Li occupation of a certain number of K transporting sites on the Na K activated ATPase with a resulting net transport of Li into the cell Recently it has been suggested that there are two K sites on this enzyme (Baker *et al* 1969) If the pumping rate of this system is controlled by the extracellular K concentration this might explain the observed effect on the Li and K content of the SRN

Our results suggest that when there is a suitable Li/K ratio Li can be transported into the SRN using the K site of the Na—K activated ATPase

Th ... ce and the
01G K69
Medicinsk
of Surrey

References

BAKER P F M P BLAUSTEIN R D KEYNES J MANIL T I SHAW and R A STEINHARDT The ouabain sensitive fluxes of sodium and potassium in squid giant axons *J Physiol* (Lond) 1969 *200* 431—458

CARLSSON B E GIACOBINI and S HOVMARK A modified microflamephotometric tecl n q e for Na and K determinations in individual somatic cells Abstr Scand Physiol Congr (Åbo) *Acta physiol scand* 1966 *68* Suppl 277 32
CARLSSON B E GIACOBINI and S HOVMARK An instrument for simultaneous determination of sodium and potassium in microsamples of biological material *Acta physiol scand* 1967 *71* 379—390
GARRAHAN P J and I M GLYNN Factors affecting the relative magn t ide of sod um potassium and sodium sodium exchanges catalysed by the sodi im pump *J Physiol* (Lond) 1967 *192* 189—216
GIACOBINI E and M STEPITA KLAUCO Studies on the mechanism of action of lithium ions I The effect of l thium ions on the impulse activity of the crayfish stretch receptor neuron *Acta physiol scand* 1970 *80* 519—527
GIACOBINI E S HOVMARK and Z KOMETIANI Intracellular variations of Na and K in isolated [illegible] 1967 71 391—400
OBARA S
fish stret
SKOU J C
Rev 1965 *45* 596—617
VAN HARREVELD A A physiological solution for fresh water crustacean *Proc Soc exp Biol* (N Y) 1936 *34* 428—432
YONEMURA K and M SATO The resting membrane potential and cation movement in frog muscle fibers after exposure to lithium ions *Jap J Physiol* 1967 *17 (6)* 678—697

Acta physiol. scand. 1970. 80 533—537
From the Department of Physiology, University of Goteborg, Sweden

Regional Distribution of Red Cells, Plasma and Blood Volume in the Intestinal Wall of the Cat

By

MATS JODAL and OVE LUNDGREN

Received 19 May 1970

Abstract

JODAL M and O LUNDGREN, *Regional distribution of red cells, plasma and blood volume in the intestinal wall of the cat* Acta physiol scand 1970 *80* 533—537

The distribution of red cells plasma and blood volume in the wall of the small intestine and in the colon was determined by means of ^{51}Cr labelled red cells and ^{125}I labelled serum albumin It was demonstrated that the submucosal tissue layer was the most vascularized Assuming a total blood content in the intestinal and colonic wall of 5 ml blood/100 g tissue the regional blood content in the submucosa could be calculated to 10—20 ml/100 g tissue while the blood content of the mucosa and muscularis amounted to approximately 3 ml/100 g

The intestine is a heterogeneous organ, its wall consisting of three well defined histological subunits, the mucosa the submucosa and the muscularis These intestinal wall layers have different functions and consequently different nutritional requirements The magnitude of the blood supply to the wall layers seems to be adjusted to their demand for oxygen and it has been demonstrated that the rate of blood flow in the "resting" mucosa of the small intestine where secretion and absorption take place is 3—5 times greater than that of the muscularis (Lundgren 1967) It therefore seems reasonable to assume that regional differences in blood volume expressed as volume per unit weight, exist within the intestinal wall In the course of some experiments, primarily designed to study plasma skimming in the intestinal tract (Jodal and Lundgren 1970) regional red cell and plasma volumes as well as blood content in the different parts of the intestinal wall were also determined These results are reported below

Methods

The [illegible] 40—60 [illegible] at least [illegible] re also

The operative procedures, nervous stimulations and isotope techniques have been descr fully in the previous publication to which the reader is referred for details. A general surve given below.

The distribution of ^{51}Cr labelled red cells and ^{125}I labelled human albumin within the intestinal wall was determined in the following way. The labelled blood was injected in venously and within 1—7 min after the injection a segment of the small intestine and/or co was extirpated and immediately frozen in a dry ice acetone mixture. The arteries and v supplying the tissue segments were simultaneously ligated just prior to the extirpation arterial blood sample was drawn via a catheter in the aortic arch when the intestinal segme were taken. The experiments were performed during three different experimental cond 1) during "resting" blood flow of the denervated intestine, 2) during vasodilatation indu by an infusion of isopropylnoradrenaline, 3) during nervous vasoconstriction.

Two flat parts from each frozen intestinal segment were selected and cut in a freeze mi tome into a number of transverse sections 100—400 μm thick from the mucosal towards serosal surface. When sectioning the tissue segments it was easy to distinguish the muco a the muscularis. The submucosal tissue was, however, seldom obtained entirely free of m and/or muscularis and since it constitutes only a small fraction of the intestinal wall (10— per cent), a comparatively large error was introduced. To indicate this the word submuc has been put in quotation marks.

Each tissue section was left in a test tube together with 2 ml concentrated sulphuric ac d at least 12 hrs for digestion of the tissue. The relative amounts of ^{125}I and ^{51}Cr, reflecting amount of plasma and red cells, respectively, were determined by means of a well type scin lation detector coupled to a spectrometer (Packard Auto-Gamma Spectrometer series 410 and a scaler.

In each experiment the relative amounts of ^{51}Cr and ^{125}I were also determined in arterial blood sample drawn at the time of extirpation of the intestinal segments (see abov The blood samples were treated in a similar way as described above. Blood hematocrit was a determined in all experiments except in a few early ones in which a value of 35 per cent w assumed. This was the mean value of all determinations made in this series of experiments.

Results

The relative distribution of red cells and plasma in the small intestine and in th colon as reflected in the distribution of ^{51}Cr and ^{125}I, respectively, is given in Tab

TABLE I. The relative distribution (mean ± SD) of ^{51}Cr labelled red cells and ^{125}I labelled seru albumin in the small intestine and the colon of the cat

	Mucosa				Submucosa				Muscularis				Organ hema tocr per cen	
	^{51}Cr red cells % of total		^{125}I plasma % of total		^{51}Cr red cells % of total		^{125}I plasma % of total		^{51}Cr red cells % of total		^{125}I plasma % of total			
Small intestine														
Rest (n = [illegible])	23	[illegible]	29	7.2	45	9.4	43	8.6	32	10.2	28	8.6	28	4.6
Vasodilatation n = 8	21	8.5	28	11.2	37	6.4	37	8.3	42	8.8	35	11.4	29	2.0
Nervous vasoconstriction (n = 9)	24	[illegible]	35	14.0	44	12.7	37	12.[illegible]	32	12.2	28	[illegible]	31	4.7
Colon														
Rest (n = 7)	[illegible]	8.7	24	7.7	47	13.7	4[illegible]	[illegible]	32	[illegible]	30	13.2	28	3.1
Vasodilatation (n = 9)	23	10.4	2[illegible]	10.[illegible]	44	11.0	41	11.1	33	11.1	34	8.2	30	4.1
Nervous vasoconstriction (n = 5)	[illegible]	11	26	11.0	51	8.2	5[illegible]	11.9	22	6.7	22	4.4	29	5.1

TABLE II Regional blood volume distribution in the small intestine and the colon of the cat

	Mucosa		"Submucosa"		Mucosa and submucosa		Muscularis	
	Blood volume, % of total	Regional blood volume, ml/100 g	Blood volume, % of total	Regional blood volume, ml/100 g	Blood volume, % of total	Regional blood volume, ml/100 g	Blood volume, % of total	Regional blood volume, ml/100 g
Small intestine								
"Rest"	27	3.0	44	21.8	71	6.4	29	3.2
Vasodilatation	26	3.9	37	25.0	63	7.7	37	5.6
Nervous vasoconstriction	31	2.3	40	12.7	71	4.2	29	2.1
Colon								
Rest	23	2.9	47	11.6	70	5.8	30	3.8
Vasodilatation	24	4.1	42	14.2	66	7.4	34	5.7
Nervous vasoconstriction	25	2.1	53	8.6	78	4.2	22	1.8

I (mean ± SD) The "organ hematocrits" are also included in this table From these values the relative distribution of the regional blood volume, expressed in per cent of the total blood volume, can be calculated (Table II)

If it is *assumed* that the "resting" blood volume of the small and large bowel wall is 5 ml/100 g tissue (see below) and that the weight relationship between mucosa, submucosa and muscularis is 45 10 45 and 40 20 40 in the small intestine and in the colon, respectively, (Jodal, Johansson and Lundgren, unpublished observations) the "resting" regional blood volume red cell volume and plasma volume can be estimated, expressed in volume per unit weight tissue The results are summarized in Table II and III

TABLE III Regional red cell and plasma volumes in the small intestine and the colon of the cat

	Mucosa		Submucosa		Mucosa and submucosa		Muscularis	
	Red cells ml/100 g	Plasma ml/100 g	Red cells ml/100 g	Plasma ml/100 g	Red cells ml/100 g	Plasma ml/100 g	Red cells ml/100 g	Plasma ml/100 g
Small intestine								
"Rest"	0.7	2.3	6.3	15.5	1.7	4.7	1.0	2.2
Vasodilatation	0.9	3.0	7.3	17.7	2.1	5.7	1.8	3.7
Nervous vasoconstriction	0.5	1.8	4.4	8.3	1.2	3.0	0.7	1.4
Colon								
"Rest"	0.7	2.1	3.3	8.3	1.6	3.9	1.1	2.7
Vasodilatation	1.1	3.0	4.5	9.7	2.3	5.2	1.7	4.0
Nervous vasoconstriction	0.6	1.5	2.5	6.0	1.2	3.0	0.5	1.3

It has been demonstrated that vasodilatation can increase the blood content of the intestinal tract 30—40 per cent (Folkow, Lundgren and Wallentin 1963 Hultén, Jodal and Lundgren 1969 b) and that nervous vasoconstrictor (4—8 imp/sec) can decrease blood content by about the same extent (Folkow *et al* 1964 Hultén *et al* 1969 b) Total blood volumes of 6 75 and 3 25 ml/100 g tissue were therefore assumed for the intestinal wall during vasodilatation and during nervous vasoconstriction respectively It was then possible to calculate the regional blood red cell and plasma volumes during these two experimental situations The results are shown in Table II (regional blood volume) and III (regional red cell and plasma volumes)

Discussion

The blood volume of the small intestine has been determined by several investigators Gibson and co workers (1946) determined the blood volume of the drained small intestine of the dog and it was found to be 4—7 ml/100 g tissue Rieke and Everett (1957) estimated the blood volume of the small intestinal wall of the anesthetized rat to be 4 7 ml/100 g tissue In a study on the cat Folkow *et al* (1963) determined the blood content of the small intestine and its mesentery thus including the large veins They arrived at a value of 7—9 ml/100 g tissue Similarly Hultén *et al* (1969 a) estimated the blood content of the colon and its mesentery to be 8—10/100 g In the light of the above mentioned studies it seemed reasonable to assume that the blood content of the intestinal wall proper of the cat amounted to approximately 5 ml/100 g

The present study strongly suggests that the submucosa contains most of the blood in the intestinal wall Expressed in volume per unit weight the blood content of the submucosa is 5—7 times that of the mucosa or the muscularis The blood volume of the submucosa in the small intestine is in fact of the same order of magnitude as that of the liver (Gibson *et al* 1946) This is in all probability explained by the existence of a dense vascular network in the submucosal tissue The blood content of the mucosa and of the muscularis is largely similar an observation that is surprising since it is known that regional blood flow in the mucosa is much larger than in the muscularis (Lundgren 1967 Hultén *et al* 1969 c It is probably explained by the fact that veins and venules draining the submucosal network are contained within the muscularis layer Thus the blood content of the muscularis proper is probably smaller than indicated by values given in Table II The points made above for blood volume also hold true for red cells and plasma

The relative distribution of blood red cells and plasma volumes (Table I and III) is similar in the two organs examined in this study Furthermore the various experimental procedures did not change the relative distribution drastically

Comparing the regional content of blood red cells and plasma in the two organs the values obtained for the mucosa and the muscularis are roughly similar in the small and large intestines The submucosal layer of the small intestine however

seems to contain almost twice as much red cells plasma and blood as the colonic submucosa when compared on a unit weight basis This finding might be explained by the observation reported above that the relative weight of the submucosa of the small intestine is only half of that of the colon

The [illegible] Johansson and Miss Lisbeth Larsson is grate- [illegible]

AIR FORCE OFFICE OF SCIENTIFIC [illegible] Aerospace Research United States Air Force under Contract No F61052 68 C-0044 by a grant from US Public Health Service (HE-05675 08) from the Swedish Medical Research Council (B70 14X 16-06B and B70-14X 2855-01) and from the Faculty of Medicine University of Goteborg

References

FOLKOW B D H LEWIS O LUNDGREN S MELLANDER and I WALLENTIN The effect of graded vasoconstrictor fibre stimulation on the intestinal resistance and capacitance vessels *Acta physiol scand* 1964 *61* 445—457

FOLKOW B O LUNDGREN and I WALLENTIN Studies on the relationship between flow resistance capillary filtration coefficient and regional blood volume in the intestine of the cat *Acta physiol scand* 1963 *57* 270—283

GIBSON J A SELIGMAN W PEACOCK I AUB J FINE and R EVANS *The distribution of red* cells and plasma in large and minute vessels of the normal dog determined by radioactive isotopes of iron and iodine *J clin Invest* 1946 *25* 848—857

HULTEN L M JODAL and O LUNDGREN Extrinsic nervous control of colonic blood flow *Acta physiol scand* 1969 a Suppl 335 39—50

HULTEN L M JODAL and O LUNDGREN Local and nervous control of the consecutive vascular sections of the colon *Acta physiol scand* 1969 b Suppl 335 51—64

HULTEN L M JODAL and O LUNDGREN Nervous control of blood flow in the parallel coupled vascular sections of the colon *Acta physiol scand* 1969 c Suppl 335 65—76

JODAL M and O LUNDGREN Plasma skimming in the intestinal tract *Acta physiol scand* 1970 *80* 50—60

LUNDGREN O Studies on blood flow distribution and countercurrent exchange in the small intestine of the cat *Acta physiol scand* 1967 Suppl 303

RIEKE W and N EVERETT Effect of pentobarbital anesthesia on the blood values of rat organs and tissues *Amer J Physiol* 1957 *188* 403—408

Acta physiol. scand. 1970. 80. 538—551

From the Laboratory for the Theory of Gymnastics, University of Copenhagen Denmark

Ventilatory Responses to Hypoxia and Hypercapnia with External Airway Resistance

By

THOMAS B BARNETT[1] and BIRGER RASMUSSEN

Received 23 May 1970

Abstract

BARNETT, T B and B RASMUSSEN *Ventilatory responses to hypoxia and hypercapnia with external airway resistance* Acta physiol scand 1970 *80* 538—551

The effects of added external airway resistance upon the steady state ventilatory response to hypoxia and hypercapnia were studied in three normal human subjects The slope of the stimulus-response line for CO_2 was reduced in all subjects when this resistance was added to the airway The ventilatory response to eucapnic hypoxia was measured in the same subjects with [illegible] to hypercapnia.

Cherniack and Snidal (1956) first demonstrated the effect of added external airway resistance upon the ventilatory response to carbon dioxide Subsequently other investigators have confirmed that the slope of the carbon dioxide ventilatory response curve is decreased in normal subjects with partial obstruction of the external airway (Eldridge and Davis 1959 Milic-Emili and Tyler 1963) Patients with obstructive airway diseases have reduced ventilatory response to carbon dioxide and when airway resistance is reduced in such patients by substituting helium for nitrogen in the respired gas mixtures there is improvement in the ventilatory response Wood and Barnett 1965 Furthermore, when the response to carbon dioxide has been expressed in terms of oxygen cost of breathing (Richards *et al* 1958) or in terms of the integrated diaphragmatic electromyogram (Lourenco and Miranda 1968) the response to carbon dioxide has been found to be similar in patients with emphysema to that in normal subjects

[1] Work done during the tenure of USPHS National Heart Institute Special Fellowship No 1-F3-HE 31638 with supplemental support from Commonwealth Fund Present address Department of Medicine University of North Carolina Chapel Hill North Carolina USA

In spite of this well established dependence of the ventilatory response to carbon dioxide upon the resistive work of breathing, the effect of increased airway resistance upon the ventilatory response to hypoxia has not, to our knowledge, been investigated This report is concerned with the effects of increasing the resistance to breathing upon the ventilatory response to hypoxia in trained normal human subjects The results are compared with the effects of the same added resistance upon the ventilatory response to carbon dioxide in the same subjects

Methods

All studies were done in 3 trained healthy young adult male subjects They reported to the laboratory in the morning 1 5 hrs after a light carbohydrate meal and rested for 30 min before beginning the first experiment These subjects had no specific knowledge of the purpose of these experiments nor were they informed of the gas mixtures used in individual experiments

[illegible]

air were made in Douglas bags and the volumes were measured in a spirometer Each collection was of approximately 35 liters, the time of collection varying with minute ventilation Corrections were made for the volume removed through the CO_2 analyser after calibrating the flow [illegible]

[illegible] ra [illegible] and [illegible]

End tidal CO_2 tension was calculated from the end expiratory plateau of the record from a Beckman [illegible] external [illegible] with [illegible] be [illegible]

[illegible] a and the alveolar equation [illegible] ary blood was sampled pH [illegible] Calibration of the CO_2 elec[illegible] the O_2 electrode was with [illegible] nitrogen an[illegible] formula ba[illegible] known gas [illegible] of equilibra[illegible]

blood was sampled from the finger after 5 minutes warming to 40 C with an electric heating pad and was carried out between the two collections of expired air Arterial samples were ob[illegible]

[illegible]

All experiments were performed with the subject supine and under steady state conditions Each experiment consisted of a 30 min exposure to the inspired gas mixture End tidal CO_2 [illegible]

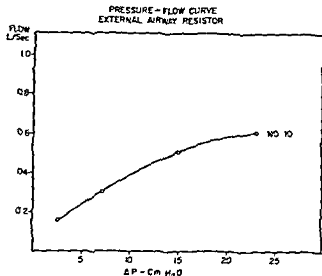


Fig 1 Pressure flow curve showing the pressure difference across the mechanical resistor used in all experiments plotted against rate of flow

The sequence of experiments with and without added airway resistance was alternated We observed an effect similar to that described by Anderton *et al* (1964) when exposure to hypoxia was repeated 30 min after the end of a prior similar exposure Because ventilation with the second hypoxia exposure was consistently greater than with identical initial exposure, we have not used in this report, any data from second experiments when first experiments were associated with alveolar oxygen tensions below 60 mm Hg Studies of this sequence effect will be reported separately

In six experiments with added airway resistance and hypoxia blood pressure was measured before during and after the experiment and vital capacity was measured before and immediately after the experiment.

Results

The data from the three subjects with and without added airway resistance during eucapnic hypoxia hypocapnic hypoxia and normoxic hypercapnia are presented in Table I, II and III

Fig 2 is a graphic presentation of the results of experiments in which the subjects breathed 10 % oxygen with end tidal P_{CO_2} maintained at the normal level In subjects GM and PB the minute ventilation was consistently lower with added external airway resistance and the alveolar oxygen tension correspondingly less In subject N.R. there was greater variation in the ventilatory response to 10 % oxygen without added resistance (se below but the average minute ventilation was less with than without added external airway resistance

Fig 3 4 and 5 depict ventilatory response curves constructed from points obtained in the steady state of hypoxia in the three subjects Each point represents the average of the duplicate volume determinations and the corresponding calculated alveolar oxygen tension at the end of a 30 min period of breathing a low oxygen mixture The curves are constructed as lines of best fit using a modified method of least squares and are not significantly different from those plotted by simple inspection The mean end tidal P_{CO_2} is shown for each curve along with the standard deviation of the

TABLE I Average of duplicate determinations on subject PB

No Added Resistance						External Resistance Added					
$\dot{V}_E$ (L/min)	f (/min)	V_T (L)	$P_{ET_{CO_2}}$ (mm Hg)	$P_{A_{O_2}}$ (mm Hg)	pH	$\dot{V}_E$ (L/min)	f (/min)	V_T (L)	$P_{ET_{CO_2}}$ (mm Hg)	$P_{A_{O_2}}$ (mm Hg)	pH
Air											
5.75	7.8	0.738	39.6	97.3		5.69	6.4	0.889	39.0	103.0	7.367
6.07	6.3	0.965	38.8	103.2	7.389	5.95	7.1	0.838	39.2	97.9	7.366
5.46	6.8	0.804	39.2	103.0	7.377	5.28	7.2	0.720	38.9	103.2	
5.72	9.4	0.609	37.9	104.4	7.337						
6.09	12.0	0.508	37.5	104.5	7.365						
6.45	10.6	0.609	37.6	99.1							
Eucapnic Hypoxia											
12.72	12.0	1.059	37.4	46.0		9.98	8.6	1.161	37.8	38.6	7.377
11.61	12.2	0.953	39.0	46.6	7.398	9.83	6.8	1.445	37.6	38.1	7.380
11.35	12.4	0.975	38.4	43.7	7.386	10.33	10.6	0.974	36.6	36.8	7.413
12.63	13.7	0.922	37.2	44.5	7.399	10.39	9.1	1.141	38.6	39.9	
11.52	12.4	0.930	38.3	44.6	7.380	7.37	8.4	0.878	37.8	52.8	
8.95	12.6	0.711	37.7	59.8	7.370	7.73	9.0	0.857	37.3	54.8	7.375
8.99	13.8	0.656	37.2	61.3	7.382	8.22	10.5	0.783	36.9	61.8	7.370
5.72	6.5	0.880	39.0	63.6							
Hypocapnic Hypoxia											
7.33	6.8	1.076	35.4	36.7	7.445	7.00	6.4	1.092	37.6	36.9	7.387
8.81	10.4	0.839	32.3	36.8	7.450	9.70	8.7	1.114	33.0	37.2	7.411
9.26	12.9	0.718	29.7	40.0		9.30	11.6	0.803	30.0	37.1	
Hypercapnia											
14.25	12.6	1.131	42.9	128.0	7.359	8.28	5.9	1.404	42.7	111.5	7.369
12.08	12.9	0.938	41.7	120.9	7.367	7.95	5.8	1.371	42.0	108.4	7.355
27.33	13.7	1.995	47.0	128.3	7.321	18.86	10.7	1.768	50.0	120.6	7.321
26.24	16.6	1.581	47.5	128.0	7.316	18.01	10.0	1.801	48.7	122.4	7.315
18.26	13.8	1.322	45.0			12.14	7.8	1.559	44.5		
19.19	14.1	1.359	43.7			12.09	6.9	1.751	45.5		

* End tidal CO_2 tension ($P_{ET_{CO_2}}$) estimated from capillary P_{CO_2}

mean The ventilatory response curve in all subjects was lower when external resistance was introduced into the airway The scatter of points about these curves varies from subject to subject and was greatest in subject NR without added resistance In this instance control of end tidal CO_2 was less satisfactory than in the other subjects and the scatter of points can be shown to be related to variations in end tidal CO_2

Fig 6, 7 and 8 show carbon dioxide stimulus-response curves for the same three subjects As in all the graphs presented here each point is established from average values for duplicate observations The lines are constructed by the method of least squares In all subjects the slope of the ventilatory response curve is much less when airway resistance has been increased artificially

Since the relationship between end tidal CO_2 and minute ventilation is a linear one within this range and since the hypoxic response curves are non linear, it is

TABLE II Average of duplicate determinations on subject NR.

No Added Resistance						External Resistance Added					
$\dot{V}_E$ (L/min)	f (/min)	V_T (L)	PET_{CO_2} (mm Hg)	PA_{O_2} (mm Hg)	pH	$\dot{V}_E$ (L/min)	f (/min)	V_T (L)	PET_{CO_2} (mm Hg)	PA_{O_2} (mm Hg)	pH
Air											
6.77	7.5	0.903	39.0	99.6	7.391	6.56	7.9	0.829	40.5*	102.0	7.394
6.59	10.0	0.659	38.4	100.2	7.384	6.26	6.0	1.043	41.5	103.0	7.353
6.50	7.5	0.867	39.4	100.6		6.06	5.7	1.062	41.6	100.4	7.395
6.80	10.7	0.635	39.5*	103.0		6.11	7.2	0.848	40.0*	101.0	7.355
Eucapnic Hypoxia											
10.36	11.4	0.909	39.6	41.2	7.375	11.01	7.0	1.575	40.2	42.9	7.385
12.07	10.2	1.182	40.4	48.5	7.363	9.60	6.0	1.600	39.1	39.3	7.400
9.69	11.9	0.816	39.4	45.3		9.20	6.2	1.482	38.3	39.8	7.385
11.48	11.2	1.025	39.5	51.0	7.374	10.33	6.8	1.518	39.4	44.2	
12.24	9.1	1.345	38.6	46.1		10.40	7.3	1.423	39.7	42.8	7.380
14.40	10.2	1.422	33.5	45.8	7.369	9.89	10.5	0.943	38.3	39.5	7.373
7.62	8.4	0.907	38.1	54.6	7.380	10.34	6.2	1.668	39.1	39.9	7.382
7.48	7.1	1.053	37.5	51.5		7.54	7.2	1.049	37.5	53.7	7.397
6.14	7.8	0.787	37.8	62.8	7.389	8.52	5.9	1.442	40.0	58.9	7.363
6.32	9.3	0.680	38.0*	64.0	7.396	6.72	6.4	1.050	38.7	63.9	7.410
						6.61	6.8	0.973	39.9	70.5	
Hypocapnic Hypoxia											
9.37	8.5	1.102	31.3	38.6	7.449	8.39	5.1	1.643	35.0	37.9	7.450
8.14	6.8	1.197	32.1	37.4	7.420	7.88	5.0	1.576	31.8	3[illegible].4	
11.38	5.0	2.239	32.0*	36.0	7.448	8.79	5.5	1.598	32.2	35.0	7.474
ypercapnia											
9.72	11.8	0.831	39.7	122.5	7.390	8.79	7.9	1.111	42.7	118.3	
11.87	10.9	1.089	41.1	118.3	7.369	[illegible].27	6.5	0.967	42.2	10[illegible].1	7.372
14.11	9.2	1.533	43.9			9.82	5.8	1.691	44.0		
28.18	11.8	2.343	48.0			14.03	7.8	1.799	47.3		
8.45	11.4	0.813	40.7			6.59	6.2	1.620	41.3		
24.12	11.2	2.178	46.0	13[illegible].3	7.334	11.97	7.5	1.596	44.8		
22.73	13.0	1.745	46.0								
10.93	10.1	1.082	41.3	115.5							

* End tidal CO_2 tension PET_{CO_2} estimated from capillary P_{CO_2}

possible to examine the relative effect of a fixed external airway resistor upon the ventilatory responses to these two stimuli by analysis of the effect on the slope of the curve. As an alternative basis for comparison the 12 liter ventilation point on each of the "no added resistance" curves has been chosen as a basis for establishing what may be considered a physiologic equivalent for these two ventilatory stimuli. The 12 liter point was chosen because of the number of actual observations made at levels of ventilation near this point in all subjects with both hypoxic and hypercapnic breathing. In each instance the level of minute ventilation corresponding to the same P_{O_2} or P_{CO_2} on the "resistance added" curve was subtracted from 12. The volume difference between the curves at this point on the horizontal axis (designated $\Delta\dot{V}_E$-12 in

TABLE III Average of duplicate determinations on subject GM

No added Resistance						External Resistance Added					
V_E (L/min)	f (/min)	V_T (L)	PET_{CO_2} (mm Hg)	PA_{O_2} (mm Hg)	pH	V_E (L/min)	f (/min)	V_T (L)	PET_{CO_2} (mm Hg)	PA_{O_2} (mm Hg)	pH
Air											
6 46	10 6	0 610	37 7	100 9	7 445	6 40	9 9	0 646	38 7	100 2	7 399
6 89	12 3	0 560	38 4	94 2	7 353	5 97	7 5	0 797	38 8	98 9	
6 87	12 4	0 553	38 9	101 0	7 371	5 85	6 1	0 961	38 5	95 4	
6 68	10 3	0 649	38 4	99 6							
6 72	11 7	0 574	37 8	98 3							
5 64	6 9	0 817	39 0*	99 6	7 392						
7 11	12 6	0 563	39 1	101 2							
Eucapnic Hypoxia											
13 49	13 5	0 999	39 1	44 6	7 370	8 89	4 1	2 168	39 3	37 2	
10 96	12 6	0 871	37 4	43 5		9 65	7 5	1 287	39 2	39 1	7 395
11 53	12 4	0 929	37 5	41 8		9 10	4 9	1 857	37 3	39 0	7 402
10 29	11 5	0 894	38 1	40 7		8 22	4 3	1 911	38 0	35 1	
10 72	13 2	0 812	39 1	40 1		8 68	4 9	1 771	38 0	37 5	
10 49	10 3	1 017	37 8	43 8		6 38	6 0	1 063	38 9	58 5	
7 85	10 7	0 734	37 9	40 9	7 388	9 07	4 7	1 930	38.5	36 7	
9 17	14 3	0 642	38 4	56 4		7 14	6 9	1 035	37 7	48 1	
7 81	11 6	0 673	38 9	54 4		7 85	7 9	0 994	39 2	54 9	7 386
						6 41	4 9	1 308	38 1	52 5	
						6 47	3 5	1 850	38 2	51 9	
						6 46	5 5	1 174	39 3	51 0	
Hypocapnic Hypoxia											
7 37	4 5	1 637	36 0*	38 0		7 95	3 5	2 272	35 5	31 5	7 412
9 34	10 8	0 865	30 8	41 6		9 19	5 8	1 585	31 6	36 0	7 483
7 89	4 0	1 973	33 0*	33 0	7 429	9 62	5 4	1 783	31 5	31 7	7 471
Hypercapnia											
10 65	13 0	0 821	40 8	112 5		8 21	8 2	1 000	42 4	114 9	7 341
13 96	14 6	0 957	42 4	124 8	7 380	6 73	5 9	1 141	42 2	107 6	7 409
9 67	12 1	0 801	41 0	115 3		11 33	4 8	2 360	47 0*	118 0	
11 92	14 6	0 818	41 1			6 21	5 6	1 109	41 9	102 9	
20 52	10 9	1 883	48 0	121 9		6 58	5 9	1 115	41 2		
10 23	11 2	0 915	41 6			6 81	6 4	1 064	41 4		

* End tidal CO_2 tension (PET_{CO_2}) estimated from capillary P_{CO_2}

TABLE IV Differences in effect of added resistance

Subject	ΔV_{E_2} (L/min)* Hypercapnia	ΔV_{E_2} (L/min) Hypoxia
GM	5 0	3 8
NR	5 1	2 5
PB	4 2	2 4

* See Figure 9

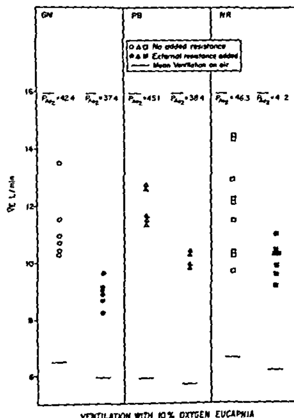


Fig 2 Minute ventilation in each of three subjects breathing 10 % oxygen without added airway resistance (open symbols) and with external resistance added to the airway (closed symbols) The mean alveolar oxygen tension for each group of experiments is shown Carbon dioxide was kept constant in all instances Mean control ventilation is shown

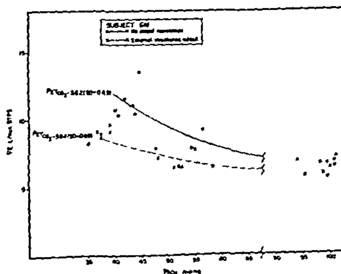


Fig 3 Ventilatory response to hypoxia in subject GM without added airway resistance (solid line) and with added resistance (broken line) The mean end tidal P_{CO_2} and standard deviation of the mean are shown for each curve

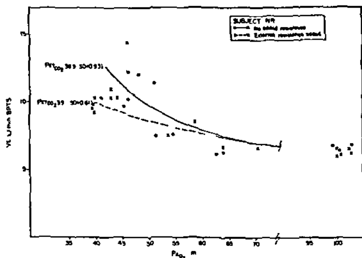


Fig 4 Ventilatory response to hypoxia in subject PB without added airway resistance (solid line) and with added resistance (broken line) The mean end tidal P_{CO_2} and standard deviation of the mean are shown for each curve

Fig 9) was used as an indication of the effect of this amount of added resistance upon the ventilatory response to that particular level of chemical stimulation Table IV shows the values for these differences By this method of comparison the difference between the two curves was greater in all of the three subjects when the principal stimulus was hypercapnia than when it was hypoxia From examining the six pairs of curves it is obvious that a similar relationship would obtain for any arbitrarily selected ventilation point on the no resistance curves below the 12 liter level

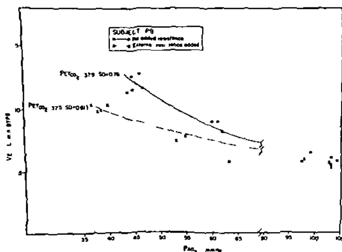


Fig 5 Ventilatory response to hypoxia in subject NR without added airway resistance (solid line) and with added resistance (broken line) The mean end tidal P_{CO_2} and standard deviation of the mean are shown for each curve

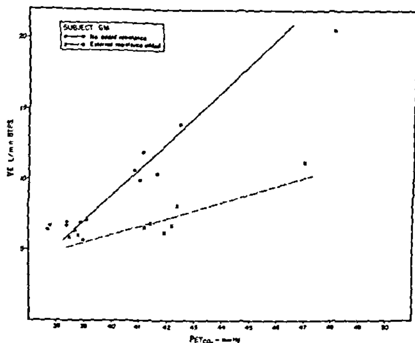


Fig 6 Ventilatory response to CO_2 in subjects GM without added airway resistance (solid line) and with added resistance (broken line)

Since end tidal CO_2 and alveolar oxygen tensions have been used throughout in the above graphs the alveolar-arterial P_{O_2} differences with and without added resistance were examined using data from 30 expts in these three subjects in which arterial samples were obtained In the presence of hypoxia the gradient was negligible (less than 1 0 mm Hg) and was the same with and without added resistance In subjects NR and PB simultaneous capillary and end tidal P_{CO_2} measurements were made in many of these as well as in other experiments In virtually all such comparative observations the end tidal P_{CO_2} was slightly less than capillary P_{CO_2} The average difference in subject NR was 1 67 mm Hg in 27 expts without added airway resistance and 1 64 mm Hg in 25 expts with added resistance In subject PB the average difference was 1 63 mm Hg in 35 experiments without added resistance and 1 25 mm Hg in 24 experiments with added resistance

The effect of added airway resistance upon respiratory frequency and tidal volume was examined from data in Table I II and III It is evident that the addition of this amount of resistance to the airway was associated with a lower respiratory frequency and a larger tidal volume than was the case with similar levels of minute ventilation without obstruction This effect was observed to a similar degree with hypoxia and hypercapnia

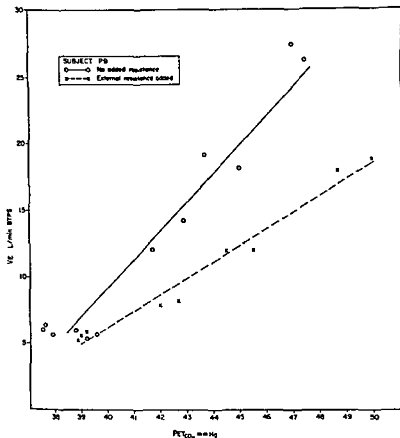


Fig 7 Ventilatory response to CO_2 in subject PB without added airway resistance (solid line) and with added resistance (broken line)

Discussion

The present investigation was concerned primarily with the effects of added external airway resistance upon the ventilatory response to hypoxia For purposes of comparison, the effects of the same amount of external airway obstruction upon the ventilatory response to carbon dioxide was studied in the same subjects In the latter experiments the results were in agreement with observations made by several other investigators (Cherniack and Snidal 1956 Eldridge and Davis 1959 Milic Emili and Tyler 1963) *i e* increased airway resistance was consistently associated with a decrease in the ventilatory response to carbon dioxide

When end tidal carbon dioxide was kept constant at near the normal level the ventilatory response to hypoxia was also lower than normal when the external airway was partially obstructed by a mechanical resistor This effect of external resistance upon the ventilatory response to eucapnic hypoxia has obvious implications with respect to the use of simple measurements of ventilation as indicators of the respon-

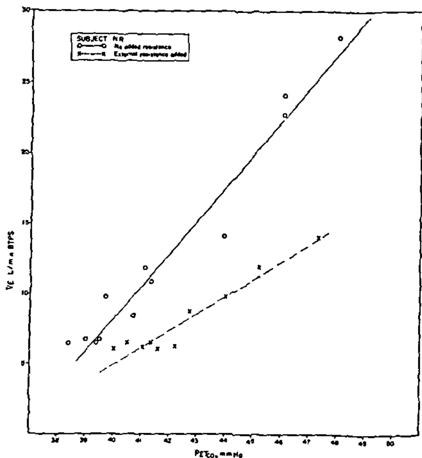


Fig 8 Ventilatory response to CO_2 in subject NR without added airway resistance (solid line) and with added resistance (broken line)

siveness or "sensitivity" of the peripheral chemoreceptors to hypoxia Some other measurement which more accurately reflects the work of breathing must be used as has been done in studying the stimulus response relationships with respect to carbon dioxide (Richards *et al* 1958 Lourenco and Miranda 1968) Recognition that the ventilatory response to hypoxia may be work dependant is especially important when studying the "sensitivity" to the hypoxic stimulus in patients with increased work of breathing

It should be emphasized that the effect of added resistive work on the ventilatory response to hypoxia has been demonstrated here only during eucapnic hypoxia In these studies carbon dioxide was added to the inspired air to maintain a constant end *tidal CO_2 tension Invariably in these experiments it required less carbon dioxide to* accomplish this when there was added resistance in the airway The question then arises as to whether the effect of added resistance on ventilation may have been in part at least an effect upon the ventilatory response to carbon dioxide or to the ele-

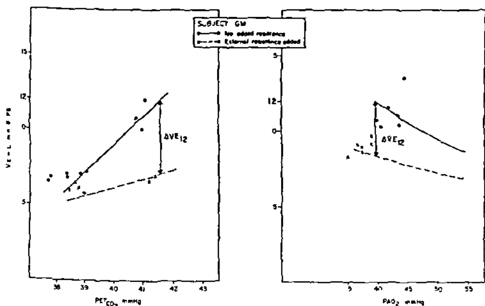


Fig 9 Portions of the ventilatory response curves from subject GM The quantity designated ΔV_{E12} in each set of curves represents the effect of the added airway resistance upon ventilation at physiologically equivalent levels of the two stimuli The magnitude of ΔV_{E12} for all subjects is shown in Table IV

ment of interaction between hypoxia and carbon dioxide Attempts to study the ventilatory response to hypoxia without correcting the hypocapnia of hypoxic hyperventilation have yielded inconclusive results The results of six experiments in each of the three subjects are included in Table II and III The relatively slight increase in ventilation under these circumstances and the variations in end tidal CO make it impossible to draw any conclusions from these experiments The level of ventilation during steady state breathing of 10 % oxygen was approximately the same with and without added airway resistance when hypocapnia was allowed to develop

Inspection of Fig 3 through 8 suggests that fixed added external airway resistance had a greater effect on the ventilatory response to hypercapnia than upon the ventilatory response to hypoxia A simple technique for comparison (see Fig 9 and Table IV) has been used in an attempt to quantitate this apparent difference and further suggests that added work does have a greater effect upon the response to hypercapnia than upon that to hypoxia in these three subjects It is obvious that the observations are too few and the scatter of points about the lines of best fit are too great to allow for final conclusions on this important matter Nevertheless it does not seem premature to examine certain possible explanations for the observation reported here

The resistor used produced greater resistance at higher flow rates (Fig 1) Since the frequency and tidal volume were similar for any given total ventilation during hypoxia and hypercapnia, it can be assu

airway resistance was essentially the same in both circumstances Whether or not the total airway resistance, including the airway resistance in the tracheobronchial system was the same, has not yet been established Hypoxia has been shown to increase air way resistance in animals (Nadel and Widdicombe 1962) Astin and Penman (1967) have recently shown a decrease in airway resistance in patients with chronic broncho-pulmonary disease when hypoxia was corrected In dogs with controlled respiration it has been shown that there is an increase in non-elastic resistance to breathing with increased arterial P_{CO_2} (Parker, Peters and Barnett 1963) Considering that the hypoxia used in the experiments reported here was rather marked and the hyper capnia necessary to give comparable ventilation was only mild one would assume that increase in internal airway resistance might be more likely during the hypoxia experiments than during the slight hypercapnia If this were so then the total airway resistance would have been changed relatively less by the addition of fixed airway resistance in the hypoxia experiments than in those with hypercapnia Such an ef fect could have contributed to the observation that the external resistance reduced the ventilatory response to hypoxia less than the response to hypercapnia In view of the high degree of resistance produced by the added resistor and the slight changes reported in hypoxic animals this does not seem to us a likely explanation for our findings We have investigated the effects of breathing 10 % oxygen on the non elastic work of breathing using three different normal young human subjects In 10 experiments we found no significant difference between the non-elastic work during hypoxic hyperventilation and the non-elastic work during a similar level of hyper capnic hyperventilation (Barnett and Smelzer unpublished data

The total contribution of stimuli from the muscles of respiration to the overall ventilatory drive is not known Campbell and Howell (1962) have demonstrated a very rapid increase in force of contraction of respiratory muscles in response to various types of added work loads a response which was too rapid to be explained by chemical stimulation due to changes in blood gas tensions They conclude that this response to added loads relates to proprioceptive reflexes arising in the muscles of the thorax While the exact mechanisms are still incompletely understood there can be no doubt about the very potent ventilatory stimulation associated with muscular exercise It seems likely that the muscles of respiration must share with other skeletal muscle groups some contribution to the total ventilatory drive especially when they are working at above normal levels against added work loads Asmussen 1967) has demonstrated a greater than additive effect upon ventilation when hypoxia is added to the exercise stimulus In earlier studies Asmussen and Nielsen (1957 showed that the combination of hypercapnia and exercise does not exhibit interaction but rather results in a total stimulus to ventilation attributable to the additive effects of the two Assuming a contributing ventilatory stimulus from the exercising respiratory mus cles when the respiratory work load is increased then it might be expected that this would be relatively greater during hypoxia than during hypercapnia Such an effect could explain at least in part the observations reported here Whether this explana tion or others as yet unknown account for these observations the data suggests that

the ventilatory response to hypoxia is less afected by the total resistive respiratory work load than is the response to hypercapnia

In certain clinical circumstances, notably in patients with chronic obstructive airway diseases, there may be a change in the relative importance of hypoxia and hypercapnia in the ventilatory drive to the extent that hypoventilation may develop when hypoxia is corrected in spite of a rise in arterial P_{CO_2} and hydrogen ion concentration. Clearly this loss of responsiveness to CO is attributable in part to mechanical factors We have shown that these same mechanical factors appear to result in a lowered ventilatory response to eucapnic hypoxia Our studies, however suggest that the effect of added resistive work may be less when hyperventilation results from hypoxia than when the same hyperventilation occurs in hypercapnic conditions Thus the present report offers evidence to suggest that the increased airway resistance itself contributes to the dissociation of the normal relationship between the hypoxic and hypercapnic drives

The authors wish to acknowledge the assistance of the staff of the Department of Biostatics University of North Carolina School of Public Health

This research was supported in part by U S Public Health Service Grants HE 01257 and FR 05406

References

ANDERTON J L E A HARRIS and K B SLAWSON The repeatability of ventilatory response to excess carbon dioxide and lack of oxygen *Quart J exp Physiol* 1964 *49* 43—51

ASMUSSEN E Exercise and the regulation of ventilation *Circulat Res* 1967 *20* Suppl I 139—145

ASMUSSEN E and M NIELSEN Ventilatory response to CO_2 during work at normal and at low oxygen tensions *Acta physiol scand* 1957 *39* 27—35

ASTIN T W and R W B PENMAN Airway obstruction due to hypoxemia in patients with chronic lung disease *Amer Rev resp Dis* 1967 *95* 567—575

CAMPBELL E J M and J B L HOWELL Proprioceptive control of breathing In Ciba Foundation Symposium *Pulmonary Structure and Function* Boston Little Brown 1962 pp 403

CHERNIACK R M and D P SNIDAL The effect of obstruction to breathing on the ventilatory response to CO_2 *J clin Invest* 1956 *35* 1286—1290

ELDRIDGE R and J M DAVIS Effect of mechanical factors on respiratory work and ventilatory

the ventilatory apparatus in 53—59

and respiratory work during *109* 908—914

NADEL J A and J G WIDDICOMBE Effect of changes in blood gas tensions and carotid sinus pressure on tracheal volume and total lung resistance to airflow *J Physiol* (Lond) 1962 *163* 13—33

PARKER J C R M PETERS and T B BARNETT Carbon dioxide and the work of breathing *J clin Invest* 1963 *42* 1362—1372

RICHARDS D W H W FRITTS JR and A L DAVIS Observations on the control of respiration in emphysema The effects of oxygen on ventilatory response to CO_2 inhalation *Trans Ass Amer Phycns* 1958 *71* 142

WOOD W B and T B BARNETT Effects of helium mixtures on ventilatory response to CO_2 in subjects with airway obstruction *Clin Res* 1965 *8* 77

Acta physiol. scand. 1970. 80. 552—566

From the Department of Physiology, Biological Institute, University of Umeå, Sweden

Discharge Patterns in Human Muscle Spindle Afferents during Isometric Voluntary Contractions

By

Å. B. VALLBO

Received 28 May 1970

Abstract

VALLBO, Å. B. *Discharge patterns in human muscle spindle afferents during isometric voluntary contractions.* Acta physiol. scand. 1970. *80.* 552—566.

Unitary nerve impulses were recorded from the median nerve of waking human subjects with percutaneously inserted tungsten electrodes. Activities from 63 muscle spindle endings were studied during isometric voluntary contractions. Three main types of response patterns were encountered: a sustained increase of the discharge, a sustained decrease, and a weak discharge consisting of a few impulses. It was concluded that the sustained increase of the discharge was accounted for by an increase of the fusimotor activity, whereas the sustained decrease was accounted for by mechanical unloading. The weak discharge might be accounted for by either mechanical or fusimotor mechanisms. Fusimotor activation concomitant with the skeletomotor ... could be demonstrated for 81 % of the spindle afferents. One particular response pattern was not characteristic for certain endings but many units responded with different patterns depending upon the intensity and spatial extent of the contraction. Analyses of the unitary responses to the contraction of different muscle portions indicated that there was a close spatial correspondence in the muscle between the skeletomotor activity and the fusimotor activity.

It was first shown by Granit and Kaada (1952) that the muscle spindle afferent discharge may be modified from the cerebral cortex through the fusimotor system. Since then fusimotor effects from this area and from the pyramidal system have been analysed in a number of investigations in the cat and the baboon (Granit 1955 b, Mortimer and Akert 1961, Shimazu, Hongo and Kubota 1962, Koeze 1968, Koeze, Phillips and Sheridan 1968, Fidone and Preston 1969, Yokota and Voorhoeve 1969, Vedel and Mouillac-Baudevin 1970). Electrical stimulation of the motor cortex may induce either an increase or a decrease of the fusimotor activity, the effect being dependent upon several factors, e.g. the level of anesthesia and whether the activity is related to flexor or extensor muscles (Granit and Kaada 1952, Koeze, Phillips and Sheridan 1968, Fidone and Preston 1969, Vedel and Mouillac-Baudevin 1970). The response of the sense organ in turn is obviously dependent upon mechanical factors in the periphery as well as on central mechanisms. It has also been shown in the primate that, under isometric conditions, the spindle response to electrical stimula-

tion of the cortex is variable in relation to the skeletomotor activity the afferent activity may increase without concomitant skeletomotor activity, the two of them may increase together, or the skeletomotor activity may increase alone without any indication of fusimotor response (Koeze 1968, Phillips and Sheridan 1968) Thus there are a number of findings indicating that a unique response pattern from muscle spindle endings is not induced by electrical stimulation of the cortex even under isometric conditions

It has also been shown that voluntary motor acts in man are associated with adjustments of the fusimotor activity Many spindle afferents respond with an increased discharge rate to isometric contractions, but a number of different patterns has been observed (Hagbarth and Vallbo 1968, 1969, Vallbo 1970) This raises the question whether different muscle spindle endings respond in different ways or else which are the factors determining the response patterns in the intact organism

In the present investigation, the discharges of spindle endings in the flexor muscles on the forearm were studied in waking human subjects It will be shown that as a rule isometric contractions were associated with a sustained increase of the afferent discharge, providing that the appropriate muscle portion was activated with a sufficient intensity The findings indicate that simultaneously with the skeletomotor activity, there was an increase of the fusimotor activity This was spatially confined to approximately the same regions of the muscle system as the skeletomotor activity

Methods

The present account is based upon the analyses of unitary discharges of 72 endings studied in 29 expts The subjects were 19 healthy adults 5 females and 14 males age between 18 and 25 years Single units were derived from the median nerve and the endings were located in the forearm muscles except one which was located in the thenar muscles For 16 of the units some observations have been presented in an earlier report Vallbo 19 0 The experimental conditions the recording technique and the recording and display systems were essentially the same as those described *in earlier papers* Vallbo *and* Hagbarth 1968 Knibestol and Vallbo 1970 Vallbo 1970) The subject was lying on a coach face downwards and his upper arm extended laterally His forearm rested on a shelf parallel to the coach The forearm and the hand was kept in pronation so that the palm was directed upwards Unitary nerve impulses were recorded with tungsten electrodes which were inserted percutaneously in the upper arm 10—15 cm above the elbow The skeletomotor activity was estimated with regard to its time course and intensity from recordings of the electromyographic activity and/or the force due to active contraction The electromyographic activity was recorded either with surface electrodes of the type described before (Vallbo 19,0 or with tungsten needles which were oated to within 4 mm from their tips The subject s hand was fixed to a device designed to allow the measurement of the twisting force and the angle at the appropriate joints Vallbo 1970 In two different series of experiments units were studied mainly from the flexor digitorum muscles or in the

well as the wrist joint angle The torque is given in meter newton Nm A few endings located in other muscles were also studied When afferent impulses related to the thumb were encountered a force due to active contraction was measured with a separate strain gauge device which was *fixed to the frame* work of the experimental set up The subject pressed the distal pad of his thumb against this strain gauge during the contractions This force which was obviously dependent upon the actual moment arm, is given in newton N For still other co

tions the amount of skeletom[illegible]

ty, as a signal related to the force was not available This was particularly the case for contractions which would rotate the hand and the forearm

The observations were done under isometric conditions The compliance of the device to which the hand was fixed and the compliance due to compression of the soft tissues of the hand taken together was equivalent to a movement at the metacarpo-phalangeal joints of 1 0—1 5 deg/Nm in the range of 0—0 5 Nm Most of the test contractions were not stronger than 0 5 Nm The wrist joint angle was either 195° or 205° i e the hand was dorsiflexed by 15° or 25°, and the angles at th[illegible] ... use joints the angles were mostly 150° or [illegible] ... lexor muscles were at their intermediate [illegible] ... ency display circuit of the type describe[illegible] ... part of the analysis As the signal to noise ratio of the nerve recordings was not very high a perfect triggering by the nerve impulses was not always obtained as might be seen in one or two of the figures A certain improvement of the signal to noise ratio was achieved by feeding the nerve signals stored on the tape recorder through a bandpass filter designed for minimum phase shift The cut off frequencies which could be changed in steps of roughly 2 dB were adjusted for optimal signal to-noise ratio for each recording As the filtering sometimes involved a pronounced decrease of the impulse amplitude it was not meaningful to give the amplitude calibration in all the records and they have been left out all through In the original records the amplitudes varied from one unit to the other between 25 and 100 microV Examples of nerve impulses which have not been filtered may be seen in earlier reports (Knibestol and Vallbo 1970 Vallbo 1970)

Essentially the same principles of selection as considered in an earlier report were followed (Vallbo 1970) Anyone unit included in the present investigation fulfilled three basic requirements it was clearly shown that the ending was located in the deep structures and not in the [illegible] ... local mechani[illegible] ... receptor with [illegible] ... A single unit [illegible] ... charging or it responded to local mechanical pressures Thus the responsiveness of the endings to voluntary contractions was not primarily a main factor of selection Many units were disregarded already during the experiments as they did not last long enough but the electrode was apparently dislocated after a minute or two There was no indication that the disregarded units had different properties than the other ones their thresholds to local stimuli and their responsiveness to voluntary contractions were not noticeably different Once the ending had been located by poking on the tissues the subject performed on instructions a number of isometric test contractions flexions of the wrist and the individual digits and rotation of the hand i e pronation and supination However all the eight types of test contractions were not systematically tested for all the units Attempts to make the subject activate smaller portions of the muscle system such as individual muscles by more detailed instructions were not tried

Results

The sensory units were separated in three groups muscle spindle units tension receptor units and units for which the receptor type was not known Sixty three units or 88 % of the total sample were classified as muscle spindle afferents on the basis of one or several of the following six criteria a sustained discharge when the muscles were relaxed and at their intermediate lengths an irregular impulse frequency when the muscles were relaxed a high dynamic sensitivity to passive joint movements a close relation between joint angle and steady state impulse frequency in relaxed muscles a poor relation between nerve impulse frequency and muscle force due to active contraction and an increase of the impulse frequency during relaxation after an isometric voluntary contraction These criteria have been discussed in detail in an earlier report (Vallbo 1970) Four units or 6 % were classified as tension receptors as their discharge frequencies during active contractions were very closely related to the force None of them exhibited any of the six muscle spindle characteristics given

above For the remaining 5 units (7 %) there was no clear indication of the receptor type and they were accordingly not classified The properties of the 63 muscle spindle units will be dealt with but the other two groups of units will not be considered further in the present report

The majority of the spindle endings were located in the wrist and finger flexor muscles of the forearm All these muscles are arranged essentially in parallel with each other It seems therefore that the probability was low for in series coupling between extrafusal fibres and muscle spindles which could give rise to an elongation of a muscle spindle during an isometric contraction On the contrary, a certain shortening would be expected, not only of the active muscle portions but also of part of the inactive ones This implies that the only mechanical effect of significance that an isometric contraction should have on any muscle spindle in the system would be to unload the spindle and thereby to lower its afferent discharge rate On the other hand a definite increase of the afferent discharge from a spindle during isometric contractions must indicate an increase of the fusimotor activity On the basis of this reasoning it seems that certain fundamental features of the fusimotor activation during voluntary motor acts could be inferred from the general discharge patterns of the spindle afferents in this muscle system

Analyses of a large number of tests indicated that the discharge patterns may con

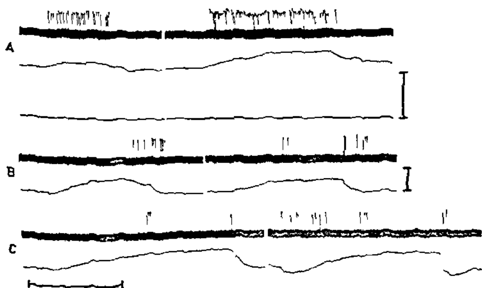


Fig 1 Re ... gers the index in A the ... n pulses analogue signals ... ulse frequency The ending ... 30 imp/sec In this and other figures upward deflections in the nerve records indicate positive signals at the electrode

veniently be separated in a few main groups, and further, that the various responses may be explained, to a large extent, on the basis of very simple assumptions Examples of different patterns are shown in Fig 1—4, 6 The records of Fig 1 were obtained during six tests in which the subject performed isometric flexions of three different fingers separately In A, B and C are depicted the responses to flexions of the index the middle finger and the ring finger respectively The contractions were approximately equally strong, as indicated by the analogue signals below the nerve records and the joint positions were the same in all the tests The six contractions were performed shortly after each other within two min It is seen that the unit responded with sustained discharges when the subject flexed his index (Fig 1 A) The first impulse appeared approximately when the force, due to active contraction started to rise and it stopped essentially when the force began to fall As will be shown later this was the most common pattern a lasting discharge during the major part of the contraction

In contrast, the responses of the same unit to flexions of the third and the fourth fingers (Fig 1 B and C) consisted of a few impulses which appeared in groups separated by long intervals It can further be seen that the responses varied to some extent from one test to the other The flexion of the fifth finger did not induce any response at all from this ending The impulse activities of Fig 1 illustrate two principle response patterns One type consisted of a discharge which lasted during the major part of the contraction (Fig 1 A) This will be referred to as a sustained increase of the discharge The other type of activity (Fig 1 B and C) was short-lasting the impulse rate was irregular and low except for occasional intervals and further it was variable from one test to the other This type will be referred to as a weak discharge The former type of activity was regarded as a conclusive evidence of increased fusimotor drive but not the latter

Responses of a continuously discharging unit are shown in Fig 2 to voluntary contractions of different muscle portions The activities in A B left and B right appeared when the middle finger the index and the ring finger were flexed re-

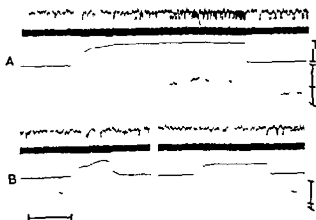


Fig 2 Responses of an ending to *isometric flexions of different* fingers the middle finger in A the index in B left and the ring finger in B right Traces indicate the same variables as in Fig 1 The ending was located 8 cm distal to the elbow in the flexor digitorum muscles

Calibrations Time 5 sec Torque 0 85 Nm Impulse frequency A 15 and 10 imp/sec B 10 and 5 imp/sec Zero levels are below scales which are cut

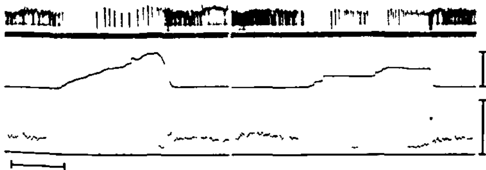


Fig 3 Responses of an ending to isometric flexions of the middle finger to the left and the ring finger to the right Same traces as in Fig 1 The ending was located 7 cm distal to the elbow in the long flexor pollicis muscle
Calibrations Time 5 sec Torque 0 3 Nm Frequency 40 imp/sec

spectively When the subject flexed his middle finger there was initially a short pause but later the impulse frequency increased and reached a higher level (12 imp/sec) than before the contraction (8 5 imp/sec) (Fig 2 A) One interpretation of this finding is that the muscle spindle was mechanically unloaded during the rising phase whereas after a few sec the intrafusal contraction grew strong enough to compensate for this effect and to increase the firing rate above the previous level This type of response was classified as a sustained increase of the discharge in spite of the initial pause When the subject flexed his index or his fourth digit the firing rate decreased (Fig 2 B) by approximately 1 0 imp/sec during part of the contraction No effect was observed when the subject flexed his fifth finger

More pronounced lowering of the impulse rate was seen in several cases An example is shown in Fig 3 from an ending located in the long flexor muscle of the thumb When the subject performed isometric flexions of his third or fourth fingers the discharge rate decreased considerably It is also seen that this effect was related not only to the force due to active contraction but in addition to the rate of change of the force as the unit stopped firing while the force was increasing but fired again when the force had reached a higher and steady level (Fig 3 right Consistently this receptor also had a high dynamic sensitivity to passive joint movements The discharge patterns illustrated in Fig 2 B and 3 will be referred to as a sustained decrease of the firing rate

An increase of the impulse rate while the force was falling at the end of a contraction was seen in association with several of the different discharge patterns as defined above Examples are shown in Fig 1 3 and 6 One interpretation is that the discharge on the falling phase of a contraction was accounted for by the passive lengthening of the spindle due to the pull by series elastic elements (*cf* Matthews 1933 Granit and van der Meulen 1962) This discharge was regarded as a separate phenomenon and it was not employed as a basis for separation of the responses in main types of patterns Another type of modification of the unitary discharges



Fig. 4. Responses of an ending to isometric flexions of the index to the left in A and B and flexion of the middle finger to the right in A and B. Traces show nerve impulses and torque due to active contractions. 30 sec are cut out between A and B. During this time the unit fired continuously.
Calibrations: Time 5 sec. Torque 0.15 Nm. The unit was located 12 cm distal to the elbow in the flexor digitorum muscles.

occasionally seen, a marked increase of the impulse rate after the isometric contraction. An example is given in Fig. 4 which shows the events associated with isometric flexions of the index and the middle finger. The unit was originally not discharging when the muscles were relaxed. On flexion of the index, the ending responded with a sustained discharge, shown to the left in A. When the subject flexed his middle finger, a few sec later, there was no response during the contraction, but afterwards a sustained discharge of low frequency appeared. This lasted until the subject performed a new flexion of his index 40 sec later, which again induced a sustained discharge during the contraction (fig. 4 B) but stopped the impulse activity after the contraction. This time a flexion of the middle finger had no effect as shown to the right in Fig. 4 B. This kind of response, consisting of a higher discharge rate after a contraction than before, was observed in 6 units. It was usually variable from one to the other and it was often more short-lasting than in Fig. 4. The mechanisms behind these responses might be either mechanical resetting of the intramuscular structures after the contraction (Granit, Homma and Matthews 1959, Koeze 1968), 'post-excitatory facilitation' of intrafusal mechanisms (Kuffler, Hunt and Quilliam 1951) or possibly a long lasting increase of the fusimotor activity.

It may be assumed that the decrease of the impulse rate shown in Fig. 2 B and 3 was accounted for by mechanical unloading, and not by a lowering of the fusimotor activity. Providing the position sensitivities of the units were known it would be possible to estimate the amount of unloading, expressed in equivalent degrees of joint movement. The unit of Fig. 2 had a position sensitivity of 0.2 imp/sec/deg wrist joint movement and it did not fire at a lower rate than 6 imp/sec. (Characteristics of this particular unit have been reported in a previous paper, Vallbo 1970, Fig. 3, 4 and 5.) The initial pause in Fig. 2 A implies that the unloading effect during the rising phase was equivalent to a wrist joint movement of at least 12.5 deg. The yield in the angle recordings and control system was negligible in this context (see p. 3). This ending had a low dynamic sensitivity and it seems therefore that this figure provides an estimate of the actual shortening of the muscle spindle. It should be stressed that a clear pause as in Fig. 2 A was seen in only a few units. However, the phenomenon is of considerable interest as it suggests that the mechanical unloading effect on the spindles may be appreciable also during weak isometric contractions

Later during the contraction in the test of Fig 2 A the impulse rate increased to a maximum of approximately 12 imp/sec This was interpreted as a result of fusimotor activity Again as the minimal discharge rate was 6 imp/sec the fusimotor activity caused an increase of the discharge rate by at least 6 imp/sec which in turn was equivalent to a wrist joint movement of 30 deg This finding indicates that the fusimotor effects associated with weak voluntary contractions are very strong compared to the effects of passive joint movements in relaxed muscles When the subject contracted neighbouring muscle portions the unloading effect was equivalent to a wrist joint movement of approximately 5 0 deg (Fig 2 B) A similar calculation for the unit of Fig 3 suggests that the maximal unloading effect was equivalent to a wrist joint movement of at least 20 deg This figure is based upon the assumption that the position sensitivity of this ending was 0 4 imp/sec/deg wrist joint movement, which was about the maximal sensitivity found among the units studied in this respect (Vallbo 1970) The acceleration of the discharge rate while the force was falling at the end of a contraction as in Fig 1, 3 and 6 seems to support the notion that there were considerable length changes of the intramuscular structures also during weak isometric contractions

It was shown above that the discharge of an ending might depend upon which portions of the muscle system the subject activated (Fig 1 and 2) This was actually true for all the units which at all responded to the test contractions One particular contraction could, as a rule be clearly defined as the most effective one in activating an ending Other contractions, engaging primarily neighbouring muscle portions gave rise to either the same pattern but a weaker response or more often a different pattern, usually a weak discharge or a sustained decrease of the discharge Contractions of even more remote muscle portions did not change the activity at all It seems reasonable to state that a receptor was functionally most closely related to those motor units which were the prime executors of that contraction which induced the most prominent discharge from the ending It is also likely that the ending actually was located in those segments of the muscle system For instance the receptors of Fig 1 and 2 were probably located in those portions of the flexor digitorum muscles which acted on the index and the middle finger respectively This interpretation was supported for many of the units by observations of their responses to passive joint movements pressure on the tendons at the wrist and the location of the ending as projected on the skin surface The muscles of origin of the unitary discharges as

TABLE I Muscles of origin of unitary spindle afferent discharges Sample 63 units

	Number of units	% of sample
Finger flexor muscles	37	59
Wrist flexor muscles	4	6
Flexor pollicis muscles	6	10
Pronating muscles	3	5
Unclear	13	21

determined in this way are given in Table I, where it can be seen that 65 per cent of the spindles studied belonged to the wrist or finger flexor muscles This was the consequence of a systematic search for the units (see p 2) Hence, the figures do certainly not reflect the relative number of spindles in the various muscles

Most clearly, the dependence of the discharge pattern upon which muscle portion the subject activated appeared from the responses of the endings located in the flexor digitorum muscles to flexions of the individual fingers Thirty endings related to the four ulnar fingers were analysed from this point of view The subject performed successively flexions of his individual fingers while the responses of the endings were studied and defined as a sustained increase of the discharge or a weak discharge as described before The contractions were approximately equally strong in any one experiment In the three separate diagrams of Fig 5, observations were collected from the endings which were most closely related to different fingers the index in A, *the middle finger in B, and the fourth finger in C The column heights give the* number of afferents in which the impulse rate increased when the subject flexed one single finger as indicated by the roman figures II—V, below each column Black areas indicate sustained increase of the discharge and white areas weak discharges. It is seen that the majority of the units responded with a sustained discharge to flexion of only one single finger (70 %), whereas the others responded with this pattern also to the flexions of one or two of the neighbouring fingers A weak discharge, on the other hand was a more common response to the flexion of neighbouring fingers Taken together, these diagrams clearly indicate that the afferent discharge from anyone spindle ending, was critically dependent upon which portion of the skeletomotor system the subject activated As considered above, it is reasonable to assume that an ending was located within that portion of the muscle system which was active when the unit responded with its maximal discharge The findings on this point therefore suggest that the fusimotor output was largely confined to the same regions as the skeletomotor output during weak voluntary contractions in this system For several reasons it could not be determined more accurately how close this spatial congruence was it was not possible to define in detail the extent of the active muscle



Fig 5 Histograms showing the distribution of the response patterns from endings in the finger flexor muscles to isometric flexions of individual fingers Observations from the units related to the index are shown in A the middle finger in B and the ring finger in C. The responses to flexions of the individual fingers are shown by the columns marked by the roman figures II for the index, III for the middle finger IV for the ring finger, and V for the little finger Black areas indicate sustained increase of the discharge white areas weak discharges as defined in the text

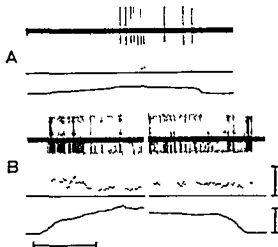


Fig 6 Responses of an ending to isometric flexions of different intensities of the thumb Traces show nerve impulses impulse frequency, and force due to active contraction The ending was located 5 cm distal to the elbow in the long flexor pollicis muscle
Calibrations Time 5 sec Force 10 N Frequency 30 imp/sec

portions in anyone contraction and, further, it was not known to what extent the weak discharges were accounted for by intrafusal contractions or by accidental mechanical effects

Another factor of significance for the discharge pattern was the intensity of the contractions As a rule, a receptor exhibited the same response pattern on repeated tests when the subject contracted the same muscle groups also when the intensities of the activations were slightly different However, for several units it could be demonstrated that a very weak contraction induced a short lasting and spurious discharge, whereas a slightly stronger contraction of the same muscle group induced a sustained discharge An example is given in Fig 6 from an ending located in the long flexor pollicis muscle The responses to flexions of different intensities of the thumb are

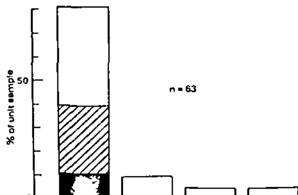


Fig 7 Percentage of units in which the principle discharge patterns in response to isometric voluntary contractions could be demonstrated A units exhibiting a sustained increase of their discharge B units exhibiting a weak discharge but not a sustained increase C units exhibiting a decrease but not a sustained increase of their discharge D units which did not respond at all to any of the test contractions The hatched area in A represents the units which in addition to the sustained increase of their discharge responded with a weak discharge and the black area represents those units which responded with a sustained increase and a sustained decrease -- different test contractions

shown A dependence of the pattern on the muscle length was noticed occasionally, but the muscle length was not varied for most of these tests

The sample records of Fig 1—3 illustrate the three principle discharge patterns encountered during isometric voluntary contractions a sustained increase of the activity a sustained decrease and a weak discharge In addition an unchanged discharge rate was of course, found in a number of tests In order to give an over all picture of the frequency of occurrence of the different patterns, the units were separated in four main groups which are presented in Fig 7 The units represented by the column A exhibited a sustained increase of the discharge in response to one or several of the test contractions It is seen that this was by far the dominating pattern which could be demonstrated for 81 % of the endings Many of these units responded in addition with another pattern under different conditions mainly when other test contractions were tried They are shown as the two subgroups of the column A The hatched area represents 29 % of the total unit sample which exhibited a weak discharge as defined above in addition to the sustained discharge as the unit of Fig 1 The black area indicates that 11 % of the units responded with a sustained decrease of the discharge besides the sustained increase as the unit of Fig 2 The column B gives the percentage of units which responded with only weak discharges This group constituted not more than 10 % of the unit sample The column C represent the units which exhibited no other response than a sustained decrease of the firing rate Obviously this kind of response could be seen only in the continuously discharging units which was 54 % of the unit sample Altogether a decrease of the discharge was seen in 30 % of the continuously discharging units Finally 5 % of the units did not respond at all during any of the contractions tested They are represented by the column D in Fig 7 All these units were silent also when the muscles were relaxed It is clear from these findings that several different discharge patterns from one and the same unit was not an exceptional finding

Discussion

One purpose of the present investigation was to define some of the basic principles according to which the fusimotor system is put into action in the intact organism As an indication of the fusimotor activity the impulse discharge from muscle spindle endings was studied (Eldred Granit and Merton 1953) The discharge during weak isometric contractions was compared with the discharge during resting conditions Isometric contractions were choosen in order to have the test conditions uncomplicated as much as possible by changes of the mechanical input to the sense organs

It was found that the impulse rates were modified in a number of different ways However the responses were separated in three main types a sustained increase of the discharge a decrease of the impulse rate and a weak discharge The most common type of response was a sustained increase of the impulse rate during the major part of the contraction This discharge was regarded as a strong evidence of fusi

motor activity since the probability for mechanical in series coupling between extrafusal fibres and spindles seemed very small in this preparation It could be demonstrated for many units that the sustained increase of the discharge occurred only when certain conditions were satisfied, the most important one being that, for any one unit a certain portion of the muscle system was activated Further, the activation had to be of sufficient intensity When these conditions were not present the discharge remained unchanged or the unit responded either with a weak discharge or the impulse rate decreased It can therefore be concluded that a certain discharge pattern, as defined in the present study, was not distinctive of certain endings but the pattern was to a large extent dependent upon the spatial and intensive characteristics of the contraction

The weak discharge consisted of a few impulses with long and irregular interspike intervals It seems likely that this kind of activity could be set up by accidental mechanical disturbances associated with an extrafusal contraction of a neighbouring muscle portion On the other hand, the activity could well be accounted for by a certain increase of the fusimotor drive, overcoming the unloading effect of the extrafusal contraction only on occasion, during a lasting contraction Both alternatives seem to be equally consistent with the finding that this response pattern was most common when the prime executor of the test contraction was a muscle portion adjacent to the one in which the receptor was located Similar findings were done concerning the sustained decrease of the discharge the contraction of one muscular portion induced an increase whereas the contraction of neighbouring portions induced a decrease The activations of more distant regions had no effect A decrease was also observed initially during slowly rising contractions These two findings are consonant with the notion that the lowering of the discharge rate was accounted for by mechanical unloading The alternative interpretation that the effect was due to a decrease of the fusimotor drive seems more remote as it would imply a rather sophisticated fusimotor outflow a decrease initially and then an increase during a lasting contraction and further a decrease to the spindles located near the active muscle portions but not to the more distant ones Thus it may be concluded in summary that a sustained increase of the discharge from the spindle endings indicated an increase of the fusimotor drive A decrease of the discharge rate was very likely accounted for by mechanical unloading whereas the spurious and weak discharge might be accounted for by either of the two

An increase of the fusimotor drive on isometric voluntary contractions could be demonstrated for the majority (81 %) of the endings This confirms earlier findings in man (Hagbarth and Vallbo 1968, Vallbo 1970) and it is largely in agreement with findings from animal experiments in which spindle afferent discharge has been analysed during natural movements or reflexly induced contractions (Granit, Job and Kaada 1952 Eldred Granit and Merton 1953, Critchlow and von Euler 1963 Davey and Taylor 1966 Severin Orlovskii and Shik 1967) The results are c sonant with the notion that alpha gamma linkage is a pronounced featur motor acts (Granit 1955 b)

However, for a number of units (19 %) an increase of the fusimotor activity could not be demonstrated during voluntary contractions Two interpretations may be suggested on this point these spindles might not be accessible for the control from motor centers engaged in voluntary contractions, or the appropriate conditions were not attained, *i e* a sufficiently strong contraction of the appropriate muscle portion was not done Several points are consonant with this last notion First, the contractions were in general very weak Second, certain portions of the muscle system might not at all have been activated during any of the test contractions as several of these contractions could be effectuated by more than one muscle For instance, the deep as well as the superficial flexor digitorum muscle could probably be used to achieve the finger flexions as done in this study (*e g* Long and Brown 1964) If the subject activated only one of these two muscles and the ending, which was studied at the moment, was located in the inactive one then the probability for acceleration of the discharge would be small (see p 9) Further, it might not be possible to reveal a small increase of the fusimotor activity to those spindles which were not discharging continuously in relaxed muscles if the fusimotor activity was not powerful enough to induce any afferent activity Considering these factors it seems reasonable to assume that a larger proportion of the unit sample would have responded with a sustained increase of the discharge if the test contractions had been varied more with regard to the intensity and the exact type of activation It is therefore even more justified to stress the finding that the majority of the spindle afferents increased their discharge rate due to fusimotor activation during voluntary contractions

) Several observations indicated that the isometric contractions gave rise to a considerable unloading effect on the muscle spindles The mechanical effect on the receptor discharge of a weak isometric contraction could be equivalent to a joint movement of 10—20 deg Hence it is obvious that the mechanical as well as the fusimotor input to the muscle spindles were changed to a large extent when the muscles contracted without joint movements In order to induce a sustained afferent discharge from the spindle endings the intrafusal contraction had to overcome the unloading effect of the concomitant extrafusal contraction This effect must be particularly pronounced on the rising phase of a contraction for those endings which have a high dynamic sensitivity It may therefore be concluded that the fusimotor drive on the spindles was stronger than suggested simply by the increase of the afferent impulse frequency over the resting discharge particularly for the primary endings at the onset of a contraction

Analysis of the unitary responses to the contractions of several neighbouring muscle portions separately indicated that a small localized skeletomotor contraction was associated with an increase of the fusimotor activity mainly to the spindles located in the same region. Thus there was a close spatial correspondance in the muscle between the activities in the skeletomotor and the fusimotor systems With regard to the central connections, on the other hand such a close spatial correspondance has not been found, as afferent impulses from the spindles in one muscle may influence the excitability of the motoneurones innervating several other muscles (Eccles Eccles

and Lundberg 1957, Eccles and Lundberg 1958 Eccles, Eccles and Shealy 1962 Willis *et al* 1966) The divergence in this respect appears to be particularly pronounced in the motor organization of the primate hand (Clough Kernell and Phillips 1968) In conformity with several other observations on animals the present findings indicate that the amount of spindle afferent activity from a contracting muscle is much greater than the activity from a relaxed muscle in the intact organism The same is obviously true for the response from the tendon organs (Jansen and Rudjord 1964, Houk and Henneman 1967) It seems therefore justified to conclude that a contracting muscle exerts a much more powerful control of the skeletomotor activity than a relaxed muscle in the complex motor system of the primate hand

This investigation was supported by grants from the Swedish Medical Research Council (Grant No B70-14X 2075 04B) The technical assistance of Mr Goran Westling is gratefully acknowledged

References

CLOUGH J F M D KERNELL and C G PHILLIPS The distribution of the monosynaptic excitation from the pyramidal tract and from primary spindle afferents to motoneurones of the baboon s hand and forearm *J Physiol* (Lond) 1968 *198* 145—166

CRITCHLOW V and C VON EULER Intercostal muscle spindle activity and its γ motor control *J Physiol* (Lond) 1963 *168* 820—847

DAVEY M R and A. TAYLOR Activity of cat jaw muscle stretch receptors recorded from their cell bodies in the mid brain during spontaneous jaw movements *J Physiol* (Lond) 1966 *185* 62—63P

ECCLES J C R M ECCLES and C N SHEALY An investigation into the effect of degenerating primary afferent fibres on the monosynaptic innervation of motoneurones *J Neurophysiol* 1962 *25* 544—558

ECCLES R. M and A LUNDBERG Integrative pattern of Ia synaptic actions on motoneurones of hip and knee muscles *J Physiol* (Lond) 1958 *144* 271—298

ELDRED E R GRANIT and P A MERTON Supraspinal control of the muscle spindles and its significance *J Physiol* (Lond) 1953 *122* 498—523

FIDONE S J and J B PRESTON Patterns of motor cortex control of flexor and extensor cat fusimotor neurons *J Neurophysiol* 1969 *32* 103—115

GRANIT R *Receptors and sensory perception* New Haven and London Yale University Press 1955 a

GRANIT R. Die Aktivierung der Muskelspindeln vom motorischen Cortex der Katze *Pflugers Arch ges Physiol* 1955 b *260* 193—196

GRANIT R S HOMMA and P B C MATTHEWS Prolonged changes in the discharge of mammalian muscle spindles following tendon taps or muscle twitches *Acta physiol scand* 1959 *46* 185—193

GRANIT R C JOB and B R KAADA Activation of muscle spindles in pinna reflex *Acta physiol scand* 1952 *27* 161—168

GRANIT R and B R KAADA Influence of stimulation of central nervous structures on muscle spindles in cat *Acta physiol scand* 1952 27 130—160

GRANIT R and J P VAN DER MEULEN The pause during contraction in the discharge of the spindle afferents from primary end organs in cat extensor muscles *Acta physiol scand* 1962 *55* 231—244

GREEN D G A transistor instantaneous frequency meter *Med biol Engng* 1967 5 387 390

HAGBARTH K E and A B VALLBO Discharge characteristics of human muscle afferents during muscle stretch and contraction *Exp Neurol* 1968 *22* 674—694

HAGBARTH [illegible] n sub-jects *Ac* [illegible]

HOUK J [illegible] of soleus mu [illegible]

JANSEN, J K S and T RUDJORD, On the silent period and Golgi tendon organs of the soleus muscle of the cat *Acta physiol scand* 1964 *62* 364—379
KNIBESTOL, M and Å B VALLBO, Single unit analysis of mechanoreceptor activity from the human glabrous skin *Acta physiol scand* 1970 *80* 178—195
KOEZE, T H, The independence of corticomotoneuronal and fusimotor pathways in the production of muscle contraction by motor cortex stimulation *J Physiol* (Lond) 1968 *197* 87—105
KOEZE, T H, C G PHILLIPS and J D SHERIDAN, Thresholds of cortical activation of muscle spindles and α motoneurones of the baboon's hand *J Physiol* (Lond) 1968 *195* 419—449
KUFFLER, S W, C C HUNT and J P QUILLIAM, Function of medullated small nerve fibres in mammalian ventral roots efferent muscle spindle innervation *J Neurophysiol* 1951 *14* 29—54
LONG, C and M E BROWN, Electromyographic kinesiology of the hand muscles moving the long finger *J Bone Jt Surg* 1964 *46 A* 1683—1706
MATTHEWS B H C, Nerve endings in mammalian muscle *J Physiol* (Lond) 1933 *78* 1—53
MORTIMER, E M and K AKERT, Cortical control and representation of fusimotor neurons *Amer J Phys Med* 1961 *40* 228—248
SEVERIN F V, G N ORLOVSKII and M L SHIK, Work of the muscle receptors during controlled locomotion *Biophysics* (eng transl of Biofizika 1967 *12* 502—511) 1967 *12* 575—586
SHIMAZU, H, T HONGO and K KUBOTA, Two types of central influences on gamma motor system J Neurophysiol 1962 *25* 309—323
VALLBO, Å B Slowly adapting muscle receptors in man *Acta physiol scand* 1970 *78* 315—333
VALLBO Å B and K E HAGBARTH, Activity from skin mechanoreceptors recorded percutaneously in awake human subjects *Exp Neurol* 1968 *21* 270—289
VEDEL, J P and J MOUILLAC-BAUDEVIN Controle pyramidal de l activite des fibres fusimotrices dynamiques et statiques chez le chat *Exp Brain Res* 1970 *10* 39—63
WILLIS W D, G W TATE R D ASHWORTH and J C WILLIS, Monosynaptic excitation of motoneurones of individual forelimb muscles *J Neurophysiol* 1966 *29* 410—424
YOKOTA T and P E VOORHOEVE Pyramidal control of fusimotor neurons supplying extensor muscles in the cat s forelimb *Exp Brain Res* 1969 *9* 96—115

Acta physiol. scand. 1970. 80. 567—576
From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Metabolic Effects of Prolonged Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue

By

Bertil B Fredholm and Jan Karlsson

Received 12 June 1970

Abstract

Fredholm, B B and J Karlsson *Metabolic effects of prolonged sympathetic nerve stimulation in canine subcutaneous tissue* Acta physiol scand 1970 *80* 567—576

The release of [illegible]

The metabolic effects of sympathomimetic catecholamines in adipose tissue has been studied extensively *in vitro* (see Hagen 1967) Adrenaline is known to increase lipolysis, glycogen breakdown glucose uptake and oxygen utilization in rat epididymal fat pads *in vitro* Sympathetic nerve stimulation in canine subcutaneous adipose tissue with an intact blood supply, increased the outflow of glycerol and free fatty acids (FFA) from adipose tissue (Rosell 1966, Fredholm and Rosell 1968), indicating an increased mobilization of stored triglycerides This effect is potentiated by adrenergic α receptor blockade and inhibited by administration of an adrenergic β receptor antagonist (Fredholm and Rosell 1968)

In the present experiments net release and uptake of glycerol, FFA, glucose, lactate, pyruvate, O_2 and CO_2 as well as tissue levels of glycogen and lactate were investigated in canine subcutaneous adipose tissue perfused with blood at a constant rate before and after sympathetic nerve stimulation Furthermore, adrenergic blocking agents, including dihydroergotamine and propranolol, have been used in an attempt to distinguish between vascular and non-vascular effects of nerve stimulation

Materials and methods

The experiments were conducted on 12 female mongrel dogs (9—24 kg, mean 15 kg) anaes-
thetized with sod [illegible]
ad [illegible]
wit [illegible]
in [illegible]
The [illegible]
[illegible] Stockholm), FFA were analyzed titrimetrically (Trout *et al* 1960) and glycerol enzymatically (Laurell and Tibbling 1966) O_2 and CO_2 were determined with the Van Slyke *method* The net uptake or release of these blood borne substrates was calculated by multiplying the A-V concentration difference by the blood or plasma flow per 100 g tissue Plasma flow was determined from hematocrit (hct) measurements The results are expressed as *μmoles taken up or released per min per 100 g tissue*, except where otherwise indicated

Biopsy specimens were [illegible]
nitrogen within 4 sec [illegible]
glucose, lactate conter [illegible]
(1970) The water co[illegible] was determined by freeze drying to constant weight

Results

The composition of arterial blood is presented in Table I Hematocrit, hemoglobin, glucose and blood gases were lower in defibrinated than in heparinized blood, presumably because the former was more diluted with saline About 25 ml saline/kg b w was administered to the dog before letting the blood for defibrination This was not necessary when heparin was administered FFA and glycerol levels were higher in heparinized blood A reason for this might be the liberation of clearing factor by heparin (Hahn 1943) A statistical analysis failed to reveal significant differences in the uptake of release of FFA glycerol glucose lactate, pyruvate or the blood gases when the tissue was perfused with the different types of blood It is thus possible to present the results without regard to the type of blood used for perfusion in the different experiments

It is also evident from Table I that the composition of the arterial blood was not constant throughout the course of the perfusion

Changes in the arterial glucose and lactate levels have been reported elsewhere

TABLE I The [illegible]

found by linear intrapolation (Fredholm 1970)

Type of blood	Time	Hct %	Hemoglobin g/100 ml	Glycerol mM	FFA mM	Glucose mM	Lactate mM	Pyruvate mM	O_2 ml/100 ml	CO_2 ml/100 ml
Def	0	29	9 2	06	30	3 3	1 4	045	12 8	36 2
	100	29	9 2	09	34	2 3+	3 0++	080	12 2	34 6+
Hep	0	40	12 4	21**	78**	5 6**	1 4	020	16 4*	41 3*
	100	40	12 3	23**	93**	4 6**+	2 0++	040	13 9+	41 4*

+ Significantly different from 0-time ($p < 0\ 05$) ++ Significantly different from 0 time ($p < 0\ 001$) * Significantly different from def blood ($p < 0\ 05$) ** Significantly different from def blood ($p < 0\ 001$)

Hypotheses concerning differences between the means were tested by means of a two-way analysis of variance (Sokal and Rohlf 1969)

(Fredholm 1970), where results showing that these changes are of little consequence for the uptake and release of metabolites are also presented

The effect of sympathetic nerve stimulation Control (Fig 1, Table II) Immediately following the start of the stimulation the perfusion pressure increased After cessation of the nerve stimulation there was a rapid drop in perfusion pressure The effects on FFA and glycerol release are similar to those reported earlier (Rosell 1966, Fredholm and Rosell 1968) Thus the release rates increased progressively after a latency period, and the maximal release rates were seen after the nerve stimulation was terminated

There was no clearcut effect on the glucose uptake whereas the pyruvate release reverted into a net uptake ($p<0\ 05$) and the lactate release increased ($p<0\ 05$) The oxygen extraction decreased significantly during the first part of the nerve stimulation (Fig 4), but returned towards prestimulatory values and was significantly higher than control after nerve stimulation CO production did not change during but increased after nerve stimulation

The lactate levels in adipose tissue biopsy specimens increased and the ratio between tissue lactate and blood lactate which probably reflects a lactate gradient was higher during nerve stimulation (Table III) The adipose tissue glycogen content decreased by 31 ± 10 per cent from a control value of $3\ 1 \pm 1\ 4$ μmoles glucose/g tissue

α receptor blockade (Fig 2 and Table II) In 5 dogs dihydroergotamine (75—150 μg i a) was administered 20 to 40 min before sampling was started The nerve stimulation caused a fall in perfusion pressure, which ended when nerve stimulation was terminated Glycerol and FFA release rates increased The latency was apparentl shorter than before receptor blockade and the peak release rates were observed ing the nerve stimulat

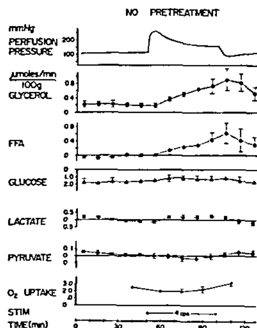


Fig 1

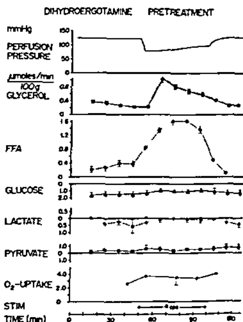


Fig 2

Fig 1 The effect of nerve stimulation (4 cps, 12 [illegible]

text

Fig 2 The effect of nerve stimulation (4 cps 12 V 2 msec for 50 min) on perfusion pressure and net uptake and release of blood borne metabolites in adipose tissue pretreated with dihydroergotamine 5 dogs Dog weight 12 10 9, 22 16 kg Adipose tissue weight 30 36 21 45 48 g *Blood flow* 0 8 1 1 0 8 1 2 1 0 ml/min Further explanation see text and legend to Fig 1

by nerve stimulation was apparently higher after dihydroergotamine administration. These findings agree with those presented earlier (Fredholm and Rosell 1968)

Nerve stimulation did not significantly affect glucose, lactate and pyruvate exchange *The oxygen increased (Fig 4) while CO_2 production was constant* Tissue lactate levels were unchanged and there was no indication of an increased lactate production (Table III) On the other hand, glycogen content was significantly lowered (26 ± 12 per cent decrease)

β-receptor blockade (Table II Fig 3) Propranolol (400 μg i a) was administered in three experiments to inhibit adrenergic β receptors The vascular response to nerve stimulation was essentially similar to that seen without drug pretreatment, and the perfusion pressure decreased below prestimulatory values when stimulation was [illegible]

lactate, pyruvate and CO_2 but the oxygen extraction fell during the first part [illegible]

TABLE II Net [illegible]

[illegible] Mean ± S.E.M.

Drug / Metabolite	No drug	Dihydroergotamine	Propranolol
Before nerve stimulation			
Glycerol	.21 ± 02	32 ± 03	21 ± 03
FFA	— 03 ± 02	42 ± 08	— 11 ± 03
Glucose	1 51 ± .26	1 45 ± 16	1 13 ± 18
Lactate	— 02 ± 06	— 48 ± 17	— 1 11 ± 28
Pyruvate	02 ± 006	03 ± 008	03 ± 004
O_2	— 2 8 ± 6	— 2 6 ± 6	— 1 6 ± 3
CO_2	3 5 ± 2 1	3 2 ± 2 1	7 ± 1 1
0—25 min stimulation			
Glycerol	30 ± 06	71 ± 17	11 ± 02
FFA	14 ± 06	1 22 ± 19	— 11 ± 03
Glucose	1 06 ± 30	1 18 ± 15	80 ± 20
Lactate	08 ± 10	— 40 ± 25	— 58 ± 22
Pyruvate	— 01 ± 028	03 ± 017	— 01 ± 016
O_2	— 2 0 ± 5	— 3 2 ± 5	— 8 ± 4
CO_2	2 6 ± 1 9	3 2 ± 2 8	1 ± 5
25—50 min stimulation			
Glycerol	53 ± 08	64 ± 05	14 ± 02
FFA	23 ± 06	1 71 ± 09	— 12 ± 02
Glucose	1 21 ± 33	84 ± 20	77 ± 12
Lactate	18 ± 06	— 20 ± 16	— 55 ± 34
Pyruvate	00 ± 036	04 ± 009	— 02 ± 009
O_2	— 2 45 ± .2	— 3 3 ± 6	— 1 6 ± 1
CO_2	3 3 ± 2 1	4 3 ± 3 8	1 1 ± 6
0—30 min poststimulatory			
Glycerol	78 ± 16	33 ± 04	48 ± 09*
FFA	66 ± 21	30 ± 20	06 ± 02*
Glucose	1 37 ± 34	1 27 ± 17	1 50*
Lactate	— 11 ± 06	— 26 ± 20	— 75*
Pyruvate	04 ± 017	08 ± 021	— 03*
O_2	— 3 5 ± 3	— 4 0	— 3 4**
CO_2	7 4 ± 7	1 2	4 2**

* 0—8 min poststim ** 8—16 min poststim S E M has been not given when n < 3

TABLE III Lactate levels in adipose tissue and venous blood before and during nerve stimulation with and without pretreatment with dihydroergotamine (400 μg i a) or dihydroergot amine (75—150 μg i a)

	Tissue lactate		Blood lactate		Tissue lactate / blood lactate	
	Before μmoles/g	Stim	Before mM	Stim.	Before	Stim
No drug	98	2 50 ($p < 0\ 05$)	1 90	2 59 ($p < 0\ 01$)	54	96 ($p < 0\ 01$)
Propranolol	67	67	1 27	1 84 ($p < 0\ 05$)	54	39
Dihydroergot	1 00	90	2 08	2 39	48	41

Statistical hypothesis testing involved Students t test for paired variates (Sokal and Rohlf

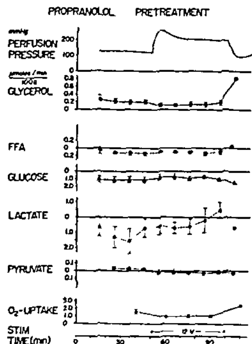


Fig 3 The effect of nerve stimulation (4 cps, 12 V, 2 msec for 50 min) on perfusion pressure and net uptake and release of blood borne metabolites in adipose tissue *pretreated with* propranolol 3 dogs Dog weight 20, 17, 16 kg Adipose tissue weight 48, 39, 72 g Blood flow 1 2, 1 0, 1 0 ml/min Further explanation see text and legend to Fig 1

nerve stimulation (Fig 4) Tissue lactate (Table III) and glycogen levels were unaltered

The total tissue water was found to be 17 ± 4 2 ml/100 g tissue in 10 biopsy specimens The glucose content was 0 5 ± 0 1 μmole/g tissue, which is at the limit of detection with the method used In the same experiments the plasma glucose level was 4 2 ± 0 8 mM The glucose space which has been taken as an index of the extracellular water space (Ballard and Hansson 1969) was 11 4 ± 2 2 ml/100 g adipose tissue

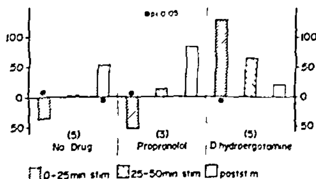


Fig 4 Changes in oxygen extraction by adipose tissue during and after nerve stimulation in per cent of control ● indicates significance at the 5 per cent level as estimated by Student's t test Figures within parenthesis denote number of experiments in each series

Discussion

The present experiments were performed to obtain information about the metabolic changes occurring in adipose tissue upon stimulation of its nervous supply. This kind of information, which appears essential to determine the role of subcutaneous adipose tissue in conditions, such as severe exercise and shock, where the sympathetic tone is elevated, was not obtainable from the literature. Furthermore, by the use of adrenergic receptor antagonists some indication of the mechanism behind the observed changes could be obtained. Stimulation of the adrenergic nerves to canine subcutaneous adipose tissue induces arteriolar and venous vasoconstriction, increased hydrodynamic conductivity, as measured by the capillary filtration coefficient, CFC (Öberg and Rosell 1967, Fredholm, Öberg and Rosell 1970), as well as decreased transport between tissue and blood (Linde and Rosell unpublished). These effects are imitated by noradrenaline and can be abolished by adrenergic *a* receptor blocking agents such as dihydroergotamine and phentolamine. The vasodilatation that occurs in this situation can be imitated by isoprenaline.

The adipose tissues were perfused at a constant rate with blood from a reservoir for three main reasons. We wanted to see whether metabolic changes in adipose tissue could be obtained in adipose tissue during nerve stimulation in spite of an unaltered total blood flow. We also wanted to secure that the changes that were observed were unrelated to changes occurring elsewhere in the body. Finally perfusion with blood from the reservoir would minimize the fluctuations in arterial blood composition.

The venous oxygen saturation averaged 77 per cent during basal (*i.e.* unstimulated) conditions, and only in two determinations in the whole series of oxygen determinations was the saturation lower than 60 per cent. It appears therefore that the blood flow provided adequate nutrition in spite of the fact that it was relatively low (2.9 ml/min/100 g in the average as compared to a mean of 6.5 obtained by Öberg and Rosell 1967 with free flow perfusion).

The nerves were stimulated with 4 imp/sec. This frequency was found by Rosell (1966) to produce maximal or near maximal rates of FFA mobilization during nerve stimulation and to cause marked vascular reactions (Ngai, Rosell and Wallenberg 1966). Folkow (1952) concluded that the sympathetic fibres in skeletal muscle of the cat might occasionally fire with a rate as high as 8 imp/sec.

The uptake and release of metabolites found in the present experiments can be compared with collected information from studies with mainly rat epididymal fat pads *in vitro* (*cf.* Hagen 1967), on the one hand, and with data obtained from a study with a similar technique as the present one on the dog gracilis muscle (Karlsson, Rosell and Saltin 1970) on the other. The glucose uptake averaged 1.4 μmoles/min/100 g which is similar to dog skeletal muscle (Karlsson *et al.* 1970) but lower than the uptake in rat epididymal fat pad *in vitro* (*e.g.* Denton *et al.* 1966). The resting oxygen uptake was about half that of canine skeletal muscle and about one tenth that reported for fat pads *in vitro* (Hagen and Ball 1961). The rate of glycerol

found in the present experiments is lower than that from adipose tissue *in vitro* including canine subcutaneous adipose tissue (Fredholm and Frisk Holmberg 1970) Whether these discrepancies are due mainly to differences in experimental technique or to species differences cannot as yet be ascertained

Tissue glycogen content averaged 3 μmoles of glucose/g tissue This value is similar to that reported by Gutman and Shafrir (1964) but higher than that reported by Denton *et al* (1966) in rat epididymal fat pads The gracilis muscle of the dog contained about 20 times more glycogen per unit weight but when expressed per ml intracellular water the two types of tissue are similar Such similarity was also demonstrated by Denton *et al* (1966) for rat muscle and adipose tissue The lactate level in adipose tissue was lower than that found in dog skeletal muscle (Karlsson *et al* 1970) when expressed per unit weight but similar when expressed per ml solvent water The lactate levels found in the present study were somewhat higher than those reported by Ballard and Hansson (1969) for rat epididymal fat pads but the blood lactate levels were also lower (about 3 times) in their study

The present results might provide some indication of the pathways of glucose metabolism in adipose tissue If it is assumed that synthesis and breakdown of glycogen balance each other in resting adipose tissue the net glucose uptake (1 4 μmoles/min/100 g) gives an estimate of the total amount of glucose available With the provisions discussed by Steinberg and Vaughan (1965) the rates of glycerol and FFA release should give an estimate of the rate of re esterification of fatty acids From the data in Table II one can calculate that about 0 6 μmoles of fatty acid are esterified per 100 g and min It is generally accepted that the glycerol moiety for triglyceride synthesis is ultimately derived from glucose (Vaughan and Steinberg 1965) Therefore about 0 1 μmole of glucose should be used for esterification If it is furthermore assumed that glucose is the major substrate for oxidation which does not appear unreasonable in view of the fed state of the dogs the oxygen extraction values from Table II would mean that 0 4 μmoles of glucose is oxidized per min per 100 g tissue Thus two thirds of the glucose taken up is left for other *e g* synthetic purposes

The glucose uptake was not significantly altered during nerve stimulation but the glycogen was broken down producing equally many glucose equivalents—provided the adrenergic β receptors were not inhibited The rate of esterification approximately doubled during and 30 min after nerve stimulation unless adrenergic receptors were inhibited in which case the rate during stimulation was similar to the pre stimulatory rate

Lactate production increased significantly during nerve stimulation and this effect was inhibited by either α or β receptor antagonists It would appear that that the lactate production depended both on an increased availability of glucose equivalents and on vasoconstriction It is of interest in this connection that oxygen extraction decreased during nerve stimulation if the vasoconstrictor response was present The lactate content in adipose tissue during nerve stimulation was 2 5 μmoles/g tissue Assuming that the lactate is distributed only in the water phase one can calculate a lactate concentration of more than 10 mM It was found recently that lactate levels

of this order of magnitude in blood significantly depressed the FFA release by increasing re-esterification (Fredholm 1970)

It is thus possible that lactate formed within adipose tissue will diminish the fatty acid mobilization, at least during prolonged nerve stimulation with frequencies of 4 cps or more With this in mind and recalling that no lactate accumulated during nerve stimulation after α blockade, it is of interest to note that the ratio between FFA and glycerol release was higher after α blockade than before

In conclusion the present results demonstrate clearcut metabolic effects of sympathetic nerve stimulation in canine subcutaneous adipose tissue in spite of an unchanged total blood flow and a constant arterial noradrenaline level The effects appear to be the sum of two separate events, namely a metabolic stimulation and a diffusion hindrance secondary to vasoconstriction

These studies were supported by the Swedish Medical Research Council (B70-14X 2203-04B B 0-40X 2553-02) The skillful technical assistance of Miss Gunilla Wikberg Miss Birgitta Edén and Miss Gun Zetterstrom is gratefully acknowledged

References

BALLARD F J and R W HANSON, Measurement of adipose tissue metabolites *in vivo* *Biochem J* 1969 *112* 195—202
BUCHER, T R. CZOK [illegible] *n der Enzymatischen Analyse* 1962 [illegible]
DENTON R. M, [illegible] ations of metabolites in adipose tiss[illegible] *Biochem J* 1966 *100* 407—419
FOLKOW B *Impulse frequency in sympathetic vasomotor fibres correlated to the release and elimination of the transmitter* *Acta physiol scand* 1952 *25* 49—76
FREDHOLM B B, The effect of lactate in canine subcutaneous adipose tissue *in situ* *Acta physiol scand* 1970 In press
FREDHOLM B B and M FRISK HOLMBERG The release of histamine and product of lipolysis from canine subcutaneous adipose tissue *Europ J Pharmacol* In press
FREDHOLM B B, B ÖBERG and S ROSELL Effects of vasoactive drugs on circulation in canine subcutaneous adipose tissue *Acta physiol scand* 1970 *79* 564—574
FREDHOLM B B. and S ROSELL Effect of adrenergic blocking agents on lipid mobilization from canine subcutaneous adipose tissue after sympathetic nerve stimulation *J Pharmacol exp Ther* 1968 *159* 1—7
GUTMAN A and E SHAFRIR Metabolic influences on glycogen synthesis and breakdown in adipose tissue *Amer J Physiol* 1964 *207* 1215—1220
HAGEN J HIMMS Sympathetic regulation of metabolism *Pharmacol Rev* 1967 *19* 367—461
HAGEN J HIMMS and E G BALL Studies of the metabolism of adipose tissue VI The effect of adrenaline on oxygen consumption and glucose utilization *Endocrinology* 1961 *69* 752—760
HAHN P F Abolishment of alimentary lipemia following injection of heparin *Science* 1943 *98* 19—20
HOHORST H J L [illegible] DPN In *Methoden der Enzymatisch* [illegible])
KARLSSON J B D [illegible] aximal and maximal exercise in man
KARLSSON J, S ROSELL and B SALTIN Carbohydrate and fat metabolism in contracting canine skeletal muscle To be published
LAURELL, S and G TIBBLING An enzymatic fluorimetric micromethod for the determination of glycerol *Clin chim Acta* 1966 *13* 317—322
NGAI S H S ROSELL and L WALLENBERG Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs *Acta physiol scand* 1966 *68* 397—403
ÖBERG B and S ROSELL Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue *Acta physiol scand* 1967 *71* 47—56

found in the present experiments is lower than that from adipose tissue *in vitro*, including canine subcutaneous adipose tissue (Fredholm and Frisk-Holmberg 1970) Whether these discrepancies are due mainly to differences in experimental technique or to species differences cannot as yet be ascertained

Tissue glycogen content averaged 3 μmoles of glucose/g tissue This value is similar to that reported by Gutman and Shafrir (1964), but higher than that reported by Denton *et al* (1966) *in rat epididymal fat pads The gracilis muscle of the dog contained* about 20 times more glycogen per unit weight, but when expressed per ml intracellular water the two types of tissue are similar Such similarity was also demonstrated by Denton *et al* (1966) for rat muscle and adipose tissue The lactate level in adipose tissue was lower than that found in dog skeletal muscle (Karlsson *et al* 1970) when expressed per unit weight but similar when expressed per ml solvent *i e* water The lactate levels found in the present study were somewhat higher than those reported by Ballard and Hansson (1969) for rat epididymal fat pads but the blood lactate levels were also lower (about 3 times) in their study

The present results might provide some indication of the pathways of glucose metabolism in adipose tissue If it is assumed that synthesis and breakdown of glycogen balance each other in resting adipose tissue the net glucose uptake (1 4 μmoles/min/100 g gives an estimate of the total amount of glucose available With the provisions discussed by Steinberg and Vaughan (1965) the rates of glycerol and FFA release should give an estimate of the rate of re-esterification of fatty acids From the data in Table II one can calculate that about 0 6 μmoles of fatty acid are esterified per 100 g and min It is generally accepted that the glycerol moiety for triglyceride synthesis is ultimately derived from glucose Vaughan and Steinberg 1965) Therefore about 0 1 μmole of glucose should be used for esterification If it is furthermore assumed that glucose is the major substrate for oxidation which does not appear unreasonable in view of the fed state of the dogs the oxygen extraction values from Table II would mean that 0 4 μmoles of glucose is oxidized per min per 100 g tissue Thus two thirds of the glucose taken up is left for other *e g* synthetic purposes

The glucose uptake was not significantly altered during nerve stimulation but the glycogen was broken down producing equally many glucose equivalents—provided the adrenergic β receptors were not inhibited The rate of esterification approximately doubled during and 30 min after nerve stimulation unless adrenergic receptors were inhibited in which case the rate during stimulation was similar to the prestimulatory rate

Lactate production increased significantly during nerve stimulation and this effect was inhibited by either α or β receptor antagonists It would appear that that the lactate production depended both on an increased availability of glucose equivalents and on vasoconstriction It is of interest in this connection that oxygen extraction decreased during nerve stimulation if the vasoconstrictor response was present The lactate content in adipose tissue during nerve stimulation was 2 5 μmoles/g tissue Assuming that the lactate is distributed only in the water phase one can calculate a lactate concentration of more than 10 mM It was found recently that lactate levels

Abstracts from Meeting of the Scandinavian Physiological Society in Stockholm 20—21 November 1970

DEMONSTRATIONS

D 1

Microelectrode Recording from the Facial Nucleus in the Cat

By C. LINDQUIST. *Department of Physiology, Karolinska Institutet, Stockholm, Sweden*

The mechanisms behind reflex discharges in the cat's facial muscles have previously been studied (Lindquist and Mårtensson 1970). Particular attention was then paid to the proprioceptive control of the muscles and the possibility was considered that exteroceptors may be engaged in the reflex control of these muscles. To be able to perform a closer analysis of the central mechanisms governing these reflexes in the cat it was considered pertinent to record the activity in the nucleus n. facialis and in associated interneuron systems.

For this purpose, lacquer insulated tungsten microelectrodes were inserted at an angle of 45° to the frontal plane through a hole in the skull. The microelectrode tip was considered to have impaled the facial nucleus when an antidromic response resulted on stimulation of a facial nerve branch and when an orthodromic action potential was set up in the facial nerve on stimulation through the electrode in this position. In some cases the exact site of the electrode was determined histologically after coagulating the nervous tissue next to the electrode tip by electric current.

Stimulation of the facial nerve 35 mm peripheral to the porus acusticus internus elicited an antidromic response in the nucleus of a latency of 1.2 msec which corresponds to a conduction velocity of about 30 m/sec. At the electrode site yielding a response of maximal amplitude a positive-negative potential of 3—4 msec duration was obtained. Similar antidromic responses of gradually decreasing amplitude were evoked over an area 2 mm above and below the site yielding the maximum response. The antidromic responses usually varied in amplitude with the stimulus strength, hence representing activity in several motoneurons, but occasionally responses of all or none type were obtained from single motoneurons.

The trigemino-facial reflex activity could also be recorded from the facial nucleus; the minimum latency observed was 4 msec.

Previous investigations (Lindquist and Mårtensson 1969, 1970) showed that reflex responses can be elicited in facial muscles on stimulation of high-threshold afferents in the facial as well as the hypoglossal nerve. In a few studies of these reflexes with microelectrodes in the facial nucleus, dispersed discharges of a latency of 20 msec could be observed. When the electrode was located in the vicinity of the ... interneurons were found which showed convergence of impulses from both

facial and hypoglossal afferents as well as from low threshold trigeminal afferents, thus possibly serving integrative functions in the facial reflexes

References

LINDQUIST C and A MÅRTENSSON Acta physiol scand 1969 77 234—240
LINDQUIST, C and A MÅRTENSSON Acta physiol scand 1970 In press

D 2

On the Dynamic Properties of the Middle Ear Reflexes

By E. BORG *Department of Physiology II, Karolinska Institutet, Stockholm, Sweden*

The acoustic stapedius and tensor tympani reflexes each form one feed back loop for the control of the sound transmission through the middle ear Their dynamic properties have been studied by recording the responses of the closed loop and the open loop systems to impulse and step function stimuli at various sound frequencies and amplitudes

The reflex activity was measured simultaneously in both ears in non anesthetized rabbits as a change in the middle ear acoustic impedance (Borg and Møller 1968) which is proportional to the integrated EMG (Borg to be published) One of the middle ear muscles had been cut or denervated several weeks before the experimental session The open loop characteristics were obtained after cutting both middle ear muscles in one of the ears The frequency spectra of the responses were calculated

) The closed loop response showed a resonance peak at about 20 Hz for the stapedius reflex and at about 12 Hz for the tensor tympani reflex The amplitude of the peak was dependent on both the frequency and the amplitude of the sound stimulus. Stimulation with high intensity sound (100 dB SPL) with a low frequency (below about 2000 Hz gave the highest peaks The open loop characteristics were also found to depend on the intensity of the sound but not on its frequency The cutoff frequency increased when the stimulus intensity increased

The open loop characteristics of the middle ear reflexes could not be described by a linear transfer function with constant coefficients since the coefficients were found to be dependent on the intensity of the sound stimulus The reflexes were instead assumed to consist of several components arranged in parallel with different thresholds The components with higher thresholds were assumed to have higher cutoff frequencies The responses of such a model turned out to be in accordance with the experimental data obtained

On the basis of the results of the experiments described above together with known data on the excitability and the sound attenuating properties of the middle ear reflexes it is possible t calculate the input to the cochlea at any arbitrary sound stimulus

References

BORG E and A R MØLLER Acta Oto-laryngologica 1968 65 575—585

D 3

Vasopressin Content in the Neurohypophysis of Rats during Overhydration and Rehydration

By N. A. Thorn and H. Vilhardt, with technical assistance of E. Engberg, I. Kjeldsen and K. Larsen. *Institute of Medical Physiology C, University of Copenhagen, Denmark*

Studies of the content of vasopressin in the neurohypophysis during depression of secretion by acute overhydration (Vilhardt 1970) seemed to indicate that hormone may be accumulated at a rate much faster than the maximum rate of secretion induced by dehydration. Repletion of hormone after a dehydration period occurs at a slower rate (Young and van Dyke 1968, Moses and Miller 1970). Since only a small series of animals were used in the studies of Vilhardt (1970), and since they had an unusually low content of vasopressin in the neurohypophysis the vasopressin content of the neurohypophysis of a larger number of rats was measured in control animals, during acute and more prolonged, forced overhydration as well as during spontaneous rehydration after a dehydration period.

The vasopressin content of the individual rats of the same group showed considerable variations.

During acute overhydration for four hours there was no significant change in the vasopressin content. Addition of ethanol to the hydration fluid had no effect. These results are at variance with those previously reported by Vilhardt (1970). During maintained massive overhydration there was a tendency to an increase in the vasopressin content after four days.

The average daily depletion of neurohypophyseal vasopressin during 5 days of dehydration was greater (about 42 mU/100 g rat) than during three days of dehydration (about 22 mU/100 g rat). On subsequent rehydration the daily increment in vasopressin content initially was faster in the group dehydrated for 5 days (about 46 versus 13 mU/100 g rat/day). It thus appears that the rate of production of vasopressin is increasing during prolonged dehydration.

References

Moses A. M. and M. Miller *Endocrinology* 1970 *86* 34—41
Vilhardt H. *Acta endocr. (Kbh.)* 1970 *63* 585—594
Young T. K. and H. B. van Dyke *J. Endocr.* 1968 *40* 337—342

D 4

Studies on Mammary Respiratory Metabolism and Mammary Development after Thyroidectomy in the Rabbit

By A NILSSON, T NILSSON and A NORGREN *Institute of Physiology and Medical Physics, University of Lund, Sweden*

Thyroid hormones have profound effects on growth and development and on respiratory metabolism in mammals (Barker 1964) Although not essential for mammary growth and differentiation, thyroid hormones may modify the mammary growth pattern *e g* in the rabbit (Norgren 1968) In view of the marked influence of thyroid hormones on respiratory metabolism of various tissues, the question was raised whether the effects of thyroid hormones on mammary development were associated with changes of the respiratory metabolism of the mammary gland

1 Thyroidectomized and shamoperated castrated rabbits were injected with ovarian hormones for 28 days The doses given stimulated optimal mammary growth and differentiation in rabbits with intact thyroid gland At autopsy oxygen consumption rates of slices of mammary gland and liver were measured with a standard Warburg technique The consumption rates (related to the DNA content of the incubated tissues) of the mammary gland were the same in thyroidectomized and shamoperated rabbits while a reduction of 30 % was found in the liver after thyroidectomy Whole mounts of glands obtained from thyroidectomized rabbits showed reduced growth and incomplete development of the alveolar system and the amount of mammary DNA was 40 % lower than that of shamoperated controls

The mammary oxygen consumption rate was lower than that observed in normal lactating mammary glands Since prolactin has well documented effects on mammary development and secretion (see Cowie 1969 Cowie *et al* 1969) our studies were extended to include mammary glands of rabbits injected with prolactin and ovarian hormones The dose of prolactin was such as to evoke a marked milk secretion in rabbits with intact thyroid gland (Gaye and Denamur 1969 Cowie *et al* 1969)

2 After thyroidectomy prolactin stimulated a marked increase of the DNA content and the oxygen consumption rates of mammary tissues The percentage increase of the two parameters was the same whether the thyroid gland was present or not The development of the mammary gland was still incomplete in the thyroidectomized rabbits injected with prolactin A small amount of milk like secretion was present Glands removed from control rabbits with intact thyroid gland were heavily distended with milk

The experiments indicate that the deficient mammary development and secretion in thyroidectomized rabbits was not associated with changes of the respiratory metabolism of the mammary tissues

References

BARKER S B "*The Thyroid Gland*" Eds PITT RIVERS and TROTTER *Vol 1* 199–23[illegible] (London 196[illegible])
COWIE A T *J Endocr* 1[illegible] 44 437 450

Cowie A T P E Hartmann and A Turvey *J Endocr* 1969 *43* 651—662
Gaye P and R Denamur *Biochim Biophys Acta* 1969 *186* 99—109
Norgren A, *Acta Univ Lund II* 1968 No 4

D 5

Studies on the Minimal Exposure Time for Androgen to Evoke Sterility in Female Rats

By T Alklint and A Norgren *Institute of Physiology and Medical Physics, University of Lund, Sweden*

Female rats injected shortly after birth with testosterone propionate, 10—30 μg or more, become sterile (Jacobsohn 1965, Barraclough 1967, Gorski 1968) It is believed that the condition is caused by an action of androgen on developing brain centers regulating reproductive functions According to *e g* Arai and Gorski (1968 a, b) the androgens would seem to exert their inductive action during a brief period, since certain substances, such as pentobarbital, phenobarbital, or the antiandrogen Cyproterone acetate, reduced the effects of testosterone propionate provided the substances were given within 6—12 hours after the administration of the androgen Under these conditions the duration of action of 1) the androgen preparation and 2) the protective substances are neglected however The minimal exposure time for androgens to cause sterility in female rats would therefore still seem to be unclear

Another method to study the minimal exposure time was used in the present work Under ether anesthesia 5 day old female rats were injected with 250 μg testosterone propionate (in 0 005 ml oil) subcutaneously into the tail The tail, and thus the depot of hormone, was removed after periods of 24 48 72 hours or longer after the injection The following parameters were recorded age at vaginal opening, age at first vaginal oestrus the incidence of persistent vaginal cornification and the incidence of sterility at 90 days or more of the rats age

Sterility was found in 80 per cent of injected control rats (tail intact) No effect on reproductive functions was observed in rats in which the hormone depot (the tail) was removed after 48 hrs When the tail was removed after 72 hrs vaginal opening and first vaginal oestrus occured significantly earlier than in uninjected control rats, and at 90 days of age constant vaginal oestrus was seen in 5 of the 10 rats studied though later 8 of them delivered young

The experiments indicate that an exposure time of more than 48 hrs is required to induce changes in the reproductive functions of 5 day old female rats injected with testosterone propionate (250 μg)

References

Arai Y and R A Gorski *Endocrinology* 1968 a *82* 1010 1014
Arai Y and R A Gorski *Proc Soc Exp Biol Med* 1968 b *127* 590—593
Barraclough C *Neuroendocrinology* 1967 2 61 99
Gorski R A *Endocrinology* 1968 *82* 1001—1004
Jacobsohn D *Acta U*[illegible] 1965 No 17

D 6

Inhibition by Prostaglandin E_1 of Vascular Response to Sympathetic Nerve Stimulation in Vivo

By P. HEDQVIST *From the Department of Physiology, Karolinska Institutet 104 01 Stockholm, Sweden*

Prostaglandin E_1 (PGE_1) has been shown to interfere with sympathetic neuromuscular transmission in the isolated cat spleen and rabbit heart, perfused with saline media as well as in the isolated guinea pig vas deferens preparation (*cf* Hedqvist 1970) The present study was designed to investigate if a similar effect of PGE_1 could be established on the vascular response to sympathetic nerve stimulation in the cat under *in vivo* conditions

Cats of both sexes were anesthetized with sodium pentobarbital (30 mg/kg i p) and given heparin (1000 I U /kg i v) and atropine (1 mg/kg i v) The adrenals were denervated and the left abdominal sympathetic trunk was dissected free and cut centrally The peripheral part of the sympathetic chain was stimulated electrically at frequencies between 1 and 5 pulses per sec at a pulse duration of 1 msec, and at supramaximal voltage The blood pressure in the left femoral artery was kept at about 100 mm Hg by means of a constant rate perfusion pump and the perfusion pressure in the left hindleg was recorded on a Grass Polygraph

Infusion of PGE_1 6×10^{-9} M into the left femoral artery produced a rapid reduction of the perfusion pressure in the hindleg Higher doses of PGE_1 up to 6×10^{-7} M increased this effect only slightly PGE_1 6×10^{-9} M slightly counteracted the pressor response to nerve stimulation The inhibition progressively increased with the dose of PGE_1 being 47 ± 3 % (mean $\pm$ S E M $n=5$) at 6×10^{-8} M Higher doses of PGE_1 up to 6×10^{-7} M caused only a slight additional inhibition of the pressor response

After the end of infusion of PGE_1 the perfusion pressure and the pressor response to nerve stimulation increased slowly and reached the control level in 5—15 min

The pressor response to noradrenaline (NA) (0 1—0 2 *μg* i a) was largely unaffected in the presence of PGE_1 (3×10^{-8} or $1\ 5\times10^{-7}$ M)

The present observations demonstrate that PGE_1 in moderate doses and under *in vivo* conditions may affect the blood pressure of perfused tissues both by a direct vasodilating action and by inhibition of the vascular response to sympathetic nerve stimulation The absence of effect of PGE_1 on the vascular response to exogenous NA strongly suggests that PGE_1 inhibits the sympathetic neurovascular system prejunctionally and acts on the process of NA release The results are compatible with and add further weight to previous considerations that PG s of the E series may play a physiologically significant regulatory role in sympathetically innervated tissues (*cf* Hedqvist 1970

References

HEDQVIST P *Acta physiol scand* 1970 Suppl 345 1 40

D 7

Biochemistry of Catecholamine Storage: A Comparison between Sympathetic Nerve Trunk and Adrenomedullary Vesicles

By K. B. Helle, H. Lagercrantz and L. Stjärne *Institute of Physiology, University of Bergen, Bergen, Norway, and Department of Physiology I, Karolinska Institutet, S-104 01 Stockholm, Sweden*

By differential and density gradient centrifugation of homogenate of bovine splenic nerve trunk a fraction has been obtained in which about 40 % of the particles represent noradrenaline (NA) storage vesicles. The purity has been checked by biochemical and morphological analysis (Lagercrantz *et al.* 1970, Klein and Thuresson-Klein 1970). In the present report results of further biochemical analysis of this fraction ("nerve trunk vesicles") are compared with observations in this and previous studies (Smith 1968) of amine storage vesicles from adrenal medulla ("adrenomedullary vesicles").

The NA content of our preparation was 3.44 μg/mg protein. Thus, by correction for contamination of our fraction it is possible to estimate the NA content of pure nerve trunk vesicles to less than 10—15 μg/mg protein. This is very low when compared with the level in adrenomedullary vesicles, 600 μg catecholamines (CA)/mg protein.

Adenosine triphosphate (ATP) was measured by the firefly method. The molar ratio NA/ATP was found to be about 7, which is considerably higher than the ratio of about 4 in adrenomedullary vesicles. During incubation of nerve trunk vesicles at 37° C for 15 min the loss of ATP was very small when compared with that of NA, and thus the NA/ATP ratio dropped sharply to 1.4. By contrast there is a parallel loss of CA and ATP in adrenomedullary vesicles. The retention of ATP in nerve trunk but not in adrenomedullary vesicles might explain why the former after depletion can be refilled with NA, while amine loss in adrenomedullary vesicles is irreversible.

Chromogranin was determined by immunodiffusion after solubilizing the pellet with triton (Banks, Helle and Mayor 1969). Less than 25 % of the chromogranin in nerve trunk vesicles was water soluble, as compared to more than 50 % in adrenomedullary vesicles (Helle and Serck-Hanssen 1969). The CA/total chromogranin ratio in nerve trunk vesicles was 310 nmoles/mg, while that in adrenomedullary vesicles was 7900 nmoles/mg.

Preliminary studies of the phospholipid composition by thin layer chromatography show certain similarities between the nerve trunk and adrenomedullary vesicles. The former were thus found to have a high content of lecithin (36 % of total phospholipid) and of phosphatidylethanolamine (34 %), close to values previously found in adrenomedullary vesicles. However, the nerve trunk vesicles seem to lack significant amounts of lysolecithin (less than 3 %), while adrenomedullary vesicles contain 16.8 % lysolecithin (Smith 1968).

These differences in the biochemistry of the amine storage particles from sy-

thetic nerve trunk and adrenal medulla suggest that there may exist important differences betwen the mechanisms of amine release during secretion *in vivo* from sympathetic nerves and adrenal medulla However, conclusions concerning secretion based on data obtained from nerve trunk vesicles must be drawn with caution in view of the uncertainty concerning the precise relationship between nerve trunk and terminal vesicles

References

BANKS P K B HELLE and D MAYOR *Mol Pharmacol* 1969 *5* 210—212
HELLE K B and G SERCK HANSSEN *Pharmacol Res Commun* 1969 *1* 25—29
KLEIN R L and A THURESSON KLEIN *Ultrastructure Res* (in press)
LAGERCRANTZ H R L KLEIN and L STJARNE *Life Sci* 1970 *9* 639—650
SMITH A D *In* Interaction of drugs and subcellular components in animal cells ed C N CAMPBELL (Churchill) London 1968 239—292

D 8

Reflex Relaxation of the Stomach Elicited from Heart Receptors

By H ABRAHAMSSON and P THOREN *Department of Physiology University of Göteborg Sweden*

Heart receptors have been supposed to be involved in the circulatory regulation in various pathophysiological situations like vasovagal syncope and myocardial infarction Since such circulatory disturbances are often associated with gastro intestinal symtoms like vomiting it was considered to be of interest to analyse whether gastric motility could be reflexly influenced from heart receptors

Heart rate and blood pressure were recorded as well as gastric motility (volume changes at constant intragastric pressure) in anesthetized cats The right cardiac nerve was mounted on electrodes for stimulation Compound action potentials were recorded from the stimulated nerve for assessment of conduction velocities

Stimulation of the cardiac nerve (4 V 1 msec 1 100 imp/sec) produced hypotension bradycardia and within 5 10 sec a marked relaxation of the stomach already with low stimulation frequencies The gastric relaxation was not due to the hypotension *per se* since it did not appear on stimulation of the aortic nerves to very low blood pressure levels Stimulation threshold and conduction velocity measurements suggested that the stimulated afferents were of the C type

Similar circulatory and gastric responses were obtained with intrapericardial injections of nicotine 25 100 μg and by partial occlusion of the ascending aorta With occlusion of the pulmonary artery no reflex responses were obtained but when the occlusion was released bradycardia and gastric relaxation appeared

The reflex relaxation of the stomach remained after administration of atropine and guanethidine but was abolished after bilateral cervical vagotomy This suggests that the efferent pathway of the reflex is the vagal relaxatory fibres to the stomach described by Martinson (1965)

It is concluded that activation of nicotine sensitive heart receptors probably

located in the ventricles and signaling through thin unmyelinated fibres produces besides circulatory effects also reflex gastric relaxation *via* the vagal relaxatory fibres From a functional point of view the receptors resemble those earlier described by Sleight and Widdicombe (1965) and those proposed to trigger the vasovagal syncope like reaction in cats (Öberg and Thorén to be published)

References

MARTINSON J *Acta physiol scand* 1965 *65* Suppl 255 1—24
SLEIGHT P and J G WIDDICOMBE *J Physiol* (Lond) 1965 *171* 235—269

D 9

Renal Vascular Resistance in Spontaneously Hypertensive Rats

By B FOLKOW, M HALLBÄCK, Y LUNDGREN and L WEISS *Department of Physiology, University of Göteborg Sweden*

Perfusion studies (Folkow *et al* 1969 1970) concerning the entire systemic vascular bed and that of the hindquarters show that flow resistance in spontaneously hypertensive rats (SHR) is increased even at *maximal* vasodilatation in proportion to the raised arterial pressure as compared to normotensive control rats (NCR) Further, resistance responses of the hindquarters at constant flow perfusion to graded noradrenaline (NA) doses show a steeper dose response curve and an increased maximal pressor response in SHR but no difference in NA threshold These findings suggest a structural change of the SHR resistance vessels where an increased media thickness encroaches upon the lumen even at maximal dilatation so that this structural change may alone explain the raised arterial pressure during rest

Since the renal vessels are of particular interest in hypertension this vascular bed was separately perfused at constant flow with oxygenated plasma substitute in 15 paired experiments on SHR and NCR At maximal vasodilatation renal resistance was if anything *lower* in SHR in contrast to the situation in other major circuits However in analogy to the hindquarter vessels there was no significant difference in NA threshold while the SHR vessels displayed a steeper dose rsponse curve crossing over that of NCR with an increased maximal pressure response The same was true for vasoconstrictors like Ba and vasopressin

These results suggest an increased media thickness also in the renal resistance vessels of SHR raising their wall/lumen ratio and maximal contractile strength However the thickened vascular walls in the kidneys seem to be combined with *increased* vascular lumina at maximal dilatation while in other circuits the wall seems to encroach upon the lumina This may imply a larger renal blood supply in SHR than in NCR at low levels of vascular tone while the reverse is true at enhanced tone due to the steeper resistance curve in SHR

References

FOLKOW B M HALLBÄCK Y LUNDGREN and L WEISS *Acta physiol scand* 1969 Su 330 94
FOLKOW B M HALLBÄCK Y LUNDGREN and L WEISS *Acta physiol scand* 1970 79

D 10

The Projection of Group I Muscle Afferents from the Hindlimb to the Contralateral Thalamus of the Cat

By S Landgren and H Silfvenius *Department of Physiology University of Umeå, Sweden*

Projections of large muscle spindle afferents from the hindlimb to the contralateral cerebral cortex of the cat were described by Landgren and Silfvenius (1969 a) The course of the ascending projection path was further analyzed in a following investigation by Landgren and Silfvenius (1969 b) The medullary relay of the path was localized to the region of the nucleus Z of Brodal and Pompeiano (1957) The location of the spinal and the thalamic relays in the path was so far unknown

Recent results reported to this meeting by Boivie Grant and Silfvenius (1970) have shown that lesions restricted to nucleus Z result in terminal degenerations in the lateral subdivision of the contralateral nucleus ventralis posterolateralis (VPLl) of the thalamus Because of this information an attempt was made to record responses evoked in VPLl by electrical stimulation of Group I muscle afferents from the hind limb Cats anaesthetized with chloralose were used for the experiments Focal potentials and extracellular action potentials from single units were recorded along penetrating microelectrode tracks The courses of the tracks were localized on histological sections

The responses evoked by the hindlimb muscle afferents were recorded in the dorsolateral border zone of the rostral part of VPLl The potentials appeared with the first sign of the afferent volley recorded from the lumbar dorsal roots The shortest latency of the thalamic response was 8 msec *i e* 3 msec shorter than the response evoked in the dorsal Group I locus of the cerebral cortex (Landgren and Silfvenius 1969 a) of the same preparation The thalamic relay was located in the zone of convergence described by Landgren Nordwall and Wengström (1965) Zonal convergence was thus observed between Group I muscle afferents low threshold knee joint and skin afferents from the hindlimb and low threshold skin afferents from the forelimb

It is concluded that the responding area is the site of a thalamic relay in the projection path from the large hindlimb muscle spindle afferents to the cerebral cortex

References

Boivie J G Grant and H Silfvenius *Acta physiol scand* 1970 00 000—000
Brodal A and O Pompeiano *J Anat* 1957 *91* 438—454
Landgren S A Nordwall and C Wengström *Acta physiol scand* 1965 *63* 164—175
Landgren S and H Silfvenius *J Physiol Lond* 1969 a *200* 353—372
Landgren S and H Silfvenius *Acta physiol scand* 1969 b Suppl 330 115

D 11

A Projection from the Nucleus Z to the Ventral Nuclear Complex of the Thalamus in the Cat

By J Boivie, G Grant and H Silfvenius *Dept of Anatomy, Karolinska Institutet, Stockholm and Dept of Physiology, University of Umeå, Sweden*

Large muscle spindle afferents from the forelimb project to the cerebral cortex *via* relays in the nucleus cuneatus and the rostromedial portion of the nucleus ventralis posterolateralis (VPL, Oscarsson and Rosen 1963 Andersson *et al* 1966 Rosén 1969) A projection to the cerebral cortex for such afferents from the hindlimb was described by Landgren and Silfvenius (1969) The medullary relay for the hindlimb afferents was localized by Landgren and Silfvenius (1969) to the region of the nucleus z of Brodal and Pompeiano (1957) but the thalamic relay has been unknown so far

The nucleus z is situated close to the dorsal surface of the medulla oblongata 3—4 mm rostral to the obex, *i e* just rostral to the nucleus gracilis Pompeiano and Brodal (1957) showed that it receives afferent fibres from the ipsilateral lateral funiculus of the spinal cord Except for the study of Brodal and Torvik (1957) indicating that the nucleus does not project to the cerebellum no previous studies have been made of the efferent projection from the nucleus z

In the present study lesions were made selectively of the nucleus z in adult cats After various survival periods the cats were perfused intracardially with formaldehyde. The degeneration was studied in silver impregnated serial transverse sections from the thalamus

The experiments showed that axons from the nucleus z enter the contralateral thalamus through the ventromedial portion of the medial lemniscus They pass rostrally just ventral and ventrolateral to the caudal part of the VPL The fibres appear to terminate in the VPL and the nucleus ventralis lateralis (VL) The terminal area in the VPL is restricted to a dorsolateral portion rostrally in the lateral part of the VPL (VPL_l), *i e* to part of the hindlimb portion of the VPL Most of the fibres to the VL terminate rostral to the VPL on the ventrolateral portion of the VL but a few of them terminate just dorsal to the rostral pole of the VPL

The findings are in full accordance with the physiological findings of Landgren and Silfvenius reported to this meeting concerning the location of the thalamic relay for the hindlimb group I muscle afferents to the VPL So far their studies have not included the VL

References

Andersson S A, S Landgren and D Wolsk *J Physiol* 1966 *183* 576—591
Brodal A and O P... *J Anat* 1957 *91* 438—454
Brodal A and A ... 7 *195* 550
Landgren S and H ... 37?
Landgren S and ... Suppl 330 119
Oscarsson O and
Pompeiano O and
Rosen I *Brain R*

D 12

Recurrent Depression from Motor Axon Collaterals of Supraspinal Inhibitory Effects in Motoneurones

By H Hultborn and M Udo *From the Department of Physiology University of Göteborg, Sweden*

Impulses in motor axon collaterals inhibit interneuronal transmission in the disynaptic Ia inhibitory pathway to motoneurones (Hultborn Jankowska and Lindström 1968) It was postulated that this recurrent control is selective, thus leaving interneurones in other reflex pathways unaffected

In the present study we have examined recurrent effects from motor axon collaterals on inhibitory transmission from the vestibulocortico and rubrospinal tracts It has been suggested that polysynaptic descending effects in motoneurones are secondary to excitation of interneurones of segmental reflex pathways (cf Lundberg 1966) If so the selective recurrent inhibition of Ia inhibitory interneurones provides a method to differentiate between IPSPs mediated by Ia inhibitory interneurones and those mediated by interneurones of other inhibitory reflex pathways The former IPSPs should thus be susceptive to recurrent inhibition while the latter should be unaffected

In all motoneurones analyzed it was controlled that Ia IPSPs were effectively depressed by ventral root volleys used to condition the descending effects

Disynaptic vestibulospinal IPSPs which can be evoked in knee flexor and some hip extensor motoneurones (Grillner Hongo and Lund 1970) were always considerably decreased by conditioning antidromic volleys The transmission of cortical IPSPs proved on several occasions to be under recurrent inhibitory control Depression of cortical IPSPs was common in flexor motoneurones while only marginal effects were observed in extensor motoneurones There was no significant recurrent depression of IPSPs evoked in extensor motoneurones from the rubrospinal tract

The regular recurrent depression of vestibulospinal IPSPs suggests that these to a great extent are mediated *via* the Ia inhibitory interneurones A considerable part of corticospinal IPSPs in flexor motoneurones seems also to be transmitted by these interneurones while cortico- and rubrospinal IPSPs in extensor motoneurones presumably are mediated by other interneurones

References

Grillner S T Hongo and S Lund *Exp Brain Res* 1970 *10* 94 120
Hultborn H E Jankowska and S Lindström *Acta physiol scand* 1968 *73* 413
Lundberg A In *Muscular Afferents and Motor Control* Nobel Symposium I Ed R Granit Almqvist & Wiksell Stockh 1966 275 305

D 13

Depression of Ia IPSP in Spinal Border Cells by Impulses in Recurrent Motor Axon Collaterals

By B GUSTAFSSON and S LINDSTROM *Department of Physiology, University of Göteborg, Sweden*

Transmission of reciprocal inhibition from muscle spindle Ia afferents to motoneurones can be inhibited at the interneuronal level by impulses in recurrent motor axon collaterals (Hultborn, Jankowska and Lindstrom 1968) Disynaptic Ia inhibition has been found also in spinal border cells (SBC), which belong to the ventral spinocerebellar system (Burke, Lundberg and Weight 1968) It will now be shown that also the Ia inhibition of SBC is susceptible to recurrent inhibition from motor axon collaterals

In cats anesthetized with chloralose Ia IPSPs were recorded intracellularly in SBC and conditioned by antidromic stimulation of different ventral roots (L5 to S1) The SBC were identified by antidromic invasion from the contralateral spinal half at low thoracic level, by their location laterally in the ventral horn and by the input from peripheral nerves (Burke *et al* 1968) As shown in Fig 1 Ia IPSPs in SBC are effectively depressed by an antidromic volley in ventral roots This was found also in cells with convergence of a monosynaptic Ia EPSP and a disynaptic Ia IPSP from the same muscle nerve (*cf* Lundberg and Weight 1970) Ia IPSPs evoked in SBC from a given nerve were depressed from the samt ventral roots as Ia IPSPs evoked in motoneurones from the same nerve The time course of the depression was also similar

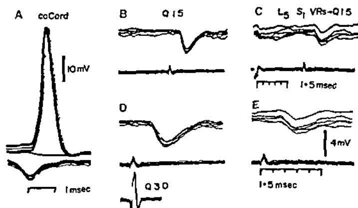


Fig 1 Recurrent depression of Ia IPSP in a spinal border cell Upper traces in A—E show intracellular respon es lower traces are from the dorsal root entry zone A antidromic spike fr (Q) ne pre- ce wit fa ol

decide whether decamethonium and carbamoylcholine share a common transport mechanism

Kidney slices were incubated in Krebs-Ringer bicarbonate medium (37° C pH 7.4) containing ^{14}C-decamethonium (2×10^{-6} M) either in presence or absence of carbamoylcholine. Each experiment was performed as a paired comparison. The uptake of decamethonium was expressed as the slice-to-medium concentration ratio (S/M ratio).

The decamethonium S/M ratio (incubation period 1 hour) was increased from 11.8 ± 0.8 to 17.3 ± 0.9 (mean $\pm$ S.E.M. of values from 6 expts.) by 10^{-3} M carbamoylcholine whereas 3×10^{-2} M carbamoylcholine decreased S/M ratio from 14.6 ± 0.5 to 7.4 ± 0.3 (mean $\pm$ S.E.M. of values from 6 expts.). Initial decamethonium uptake (S/M ratio after incubation for 3 minutes) was increased from 1.11 ± 0.04 to 1.31 ± 0.06 *(mean $\pm$ S.E.M. of values from 6 expts.)*, when slices were preincubated (1 hr) with 3×10^{-3} M carbamoylcholine before transfer to a carbamoylcholine free medium containing decamethonium. The results suggest a relationship between the stimulation phenomenon and the presence of carbamoylcholine in the slices. Efflux of decamethonium from slices incubated (1 hr) with 2×10^{-6} M ^{14}C-decamethonium before transfer to a decamethonium free medium was investigated. No efflux of decamethonium into the latter medium was observed during an incubation period of 15 min. which means that the increased uptake cannot be attributed to inhibition of decamethonium efflux by carbamoylcholine.

The stimulating effect of carbamoylcholine on decamethonium uptake is interpreted as an example of accelerative exchange diffusion.

References

HOLM, J.: *Acta pharmacol. et toxicol.* 1970 a. *28.* 192—202.
HOLM, J.: *Acta pharmacol. et toxicol.* 1970 b. *28.* 370—377.
HOLM, J.: *Acta pharmacol. et toxicol.* 1970 c. *28.* Suppl. 1. 51.

D 17

Effect of Training with Eccentric Muscle Contractions on Human Skeletal Muscle Metabolites

By F. BONDE PETERSEN and H. G. KNUTTGEN. *Department of Physiology, Gymnastik- och Idrottshögskolan, Stockholm, Sweden*

It was shown by Knuttgen and Klausen (1970) that a period of training with eccentric muscle contractions during bicycling resulted in a decrease in oxygen consumption during prolonged (1.2–1 hr) periods of eccentric exercise. One of the hypotheses for the explanation of this phenomenon was that the muscle cell was trained to accept mechanical energy due to adaptation of the enzymatic system of the cell, resulting in a decrease in the oxygen necessary to produce the same muscle tension after the training period.

In order to investigate this possibility muscle biopsies were taken with the

technique (Bergstrom 1962) from the m vastus lateralis before and after 4 weeks of eccentric training on a bicycle ergometer (Petersen 1969) Six young male subjects trained 5 days a week for 1/2 hr each day with eccentric bicycle exercise at intensities equal to 70 80 or 90 per cent of the intensity necessary to elicit maximum oxygen consumption during concentric bicycle exercise The biopsies were taken at rest, after 4 min and after 30 min of eccentric exercise and immediately (within 8 sec) frozen in fluid nitrogen The muscle samples were analyzed for ATP CP glycogen glucose 6-P and lactate During the test work heart rate oxygen consumption (Douglas-bag and Haldane analysis) and RQ were measured at frequent intervals In addition a subjective rating of the work intensity according to Borg (1962) was made at 5 min intervals *Before and after the training period a jump test was* performed and the isometric strength in leg extension was tested

During the eccentric exercise there was no changes in ATP while the CP decreased from 17 0 to 14 6 mmoles × kg [1] wet muscle before training and from 17 0 to 14 3 mmoles × kg [1] wet muscle after training In both of the two exercise tests the glycogen showed *a tendency* to a decrease due to the 30 min eccentric exercise while the lactate and the glucose 6-P content of the muscle samples showed a tendency towards an increase There was no difference in isometric muscle strength during leg extension or in the results of the jump test due to training However the oxygen consumption and the heart rate during eccentric exercise decreased after training as also found by Knuttgen and Klausen (1970) The subjective rating of the work intensity decreased in parallel to the decrease in heart rate

It was concluded that the present experiment did not show any evidence of enzymatic adaptation as a result of eccentric muscle training

References

BERGSTROM J *Scand J Clin Lab Invest* 1962 *14* Suppl 68 1—110
BORG G A V Physical Performance and Perceived Exertion Lund 1962
KNUTTGEN H G and K KLAUSEN *J Appl Physiol* 1970 Submitted
PETERSEN F B *Int Z angew Physiol* 1969 27 133—137

D 18

Energy Metabolism during Isometric Exercise at Different Temperature of M Quadriceps Femoris in Man

By R H T EDWARDS * R C HARRIS E HULTMAN L KAIJSER D KOH and L O NORDESJO *Military Medical Examination Center (MMUC) Department of Clinical Physiology Karolinska sjukhuset Department of Clinical Chemistry S t Eriks sjukhus Stockholm*

In nine healthy male volunteers the temperature of m quadriceps femoris was altered by immersion of a leg in a water bath at 12° 26° or 44° C for 45 min Corresponding final mean muscle temperatures (measured at 3 sites in one subject each) were 22 5

* In receipt of a Swedish Wellcome Travelling Research Fellowship

31 6 and 38 6° C respectively Starting immediately after leaving the water bath seven isometric contractions were sustained to fatigue at 20 sec intervals while the subject was seated in an adjustable chair (Tornvall 1963) Muscle tension (measured with a dynamometer situated at the ankle when the knee was at 90°) was maintained at 70 % of Maximum Voluntary Contraction In 6 subjects muscle biopsies (Bergstrom 1962) were taken before and at the end of the 1st and at the end of the 2nd and 7th contractions Holding times averaged 65 6 (±6 8 SEM), 27 5 (±1 9 SEM) and 16 5 (±3 4 SEM) seconds for the 1st 2nd and 7th contraction respectively with a water bath temperature of 26° C Times were slightly but not significantly shorter with 12° C but with 44° C holding times were markedly reduced (in agreement with Clarke *et al* 1958) averaging 41 5 (±4 4 SEM) 15 7 (±1 9 SEM) and 11 7 (±2 6 SEM) sec respectively

Increase in muscle temperature accelerated glycolysis as evidenced by changes in muscle metabolites measured enzymically (after Lowry *et al* 1964) and the supply of energy from Creatine Phosphate and Adenosine Triphosphate (ATP) Fluxes of high energy phosphate (estimated for the first contraction) averaged 252 (±54 SEM) 219 (±34 SEM) and 503 (±54 SEM) µmol/100 g dry weight/sec with 12°, 26° and 44° C respectively

Shortened holding times by heated muscle could be due to less efficient utilization of ATP in maintaining the isometric contraction with greater random fluctuation in sarcomere length (Goldspink *et al* 1970)

References

BERGSTRÖM J *Scand J Lab Clin Invest* 1962 *14* Suppl 68 11—13
CLARKE R S J R F HELLON and A R LIND *J Physiol* (Lond) 1958 *143* 454—473
GOLDSPINK G R F LARSON and R E DAVIES *Experientia* 1970 *26* 16—18
LOWRY O H J V PASSONNEAU F HASSELBERGER and D W SCHULZ *J Biol Chem* 1964 *239* 18—30
TORNVALL G *Acta physiol scand* 1963 *58* Suppl 201 21—36

C 1

Ventilatory Response to CO_2 in Hyperbaric Environments

By L. FAGRAEUS and C M HESSER *Department of Naval Medicine Karolinska Institutet, Stockholm, Sweden*

The reduced ventilatory response to muscular exercise observed at high ambient air pressures is due to the combined effects of raised inspired O_2 pressure and other factors (Hesser, Fagraeus and Linnarsson 1968) Theoretically, there are at least two such nonoxygen dependent' factors, namely (I) increased work of breathing due to the increased gas density, and (II) a narcotic action of high N_2 pressures on neural structures involved in the control of respiration

In an attempt to separate and evaluate the relative importance of these two potential factors, the ventilatory response to CO_2 (V_E/P_{ACO_2}) was determined in eight experienced divers under two different conditions *i e*, when breathing low concentrations of CO_2 in oxygen at 1 7 atmospheres absolute (ATA) and in air at 8 0 ATA The inspired O_2 and CO_2 pressures were approximately the same in the two conditions, whereas the inspired N_2 pressures differed by 6 3 ATA The density of the inspired gas mixtures at 8 0 ATA was about 4 2 times that of the mixtures at 1 7 ATA

As shown in Fig 1, a rise in N_2 pressure from 0 to 6 3 ATA caused an almost 50 per cent reduction in slope (ΔV_E 1 min$^{-1}/\Delta P_{ACO_2}$, mm Hg) with no conspicuous shift in position of the CO_2 response curve Since curves obtained from subjects with added breathing resistance also show mainly a reduction in slope (Cherniack 1965 Milic Emili and Tyler 1963) whereas those from subjects under conventional anesthesia show both a reduction in slope and a shift to the right (Fink *et al* 1963) it may be concluded that the reduction of ventilation observed in the present study was

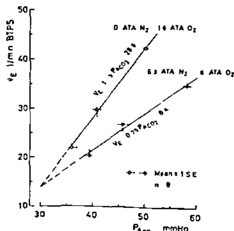


Fig 1 Effects of changes in inspired P_{N_2} and gas density on the ventilatory response to CO_2 with inspired P_{O_2} kept constant *Open circles* Data obtained at 1 7 ATA with subjects breathing various CO_2—O_2 mixtures *Closed circles* Data obtained at 8 0 ATA with subjects breathing various CO_2-air mixtures Inspired CO_2 concentrations equivalent to approximately 2 4 and 6 per cent at sea level pressure Ratio of density of gas mixtures inspired at 8 0 and at 1 7 ATA approximately 4 2 1 One standard error above and below the mean value is shown both for respiratory minute volume (V_E) and for alveolar P_{CO_2} (P_{ACO_2})

due to the increase in gas density and breathing resistance rather than to any narcotic action of the high N_2 pressure By inference, most of the nonoxygen dependent reduction of the ventilatory response to exercise occurring at high ambient air pressures may be ascribed to the increased work of breathing due to the increase in gas density

References

CHERNIACK R M *Handbook of Physiology, Section 3 Respiration II* Washington D C Am Physiol Soc 1965 Chapt 60, pp 1469—1474
FISK B R E C HANKS S H NGAI and E M PAPPER, *Ann N Y Acad Sci* 1963 *109* 892—899
HESSER C M L FAGRAEUS and D LINNARSSON *Proc Intern Union Physiol Sci* 1968 *7* 191
MILIC EMILI J and J M TYLER *Ann N Y Acad Sci* 1963 *109* 908—914

C 2

Regulation of Depth and Rate of Breathing in Cat and Man

By F J CLARK and C von EULER. *Nobel Institute for Neurophysiology Karolinska Institutet Stockholm*

The sequence of events in a breathing cycle in terms of inspiratory time expiratory time and volume has been studied Experimental data from cats (pentobarbitone) and man were obtained from rebreathing trials and from cats by measuring the effects of brief inflations applied at various times during the respiratory cycle The from breath by breath measurements were analyzed by digital computer (IBM 1800

The relationship between inspiratory volume (V_I) and inspiratory time or duration (T_I) appeared governed by at least two distinct mechanisms which operated over different volume ranges as shown in Fig 1 The duration of the expiratory phase (T_E) appears to be set simultaneously with T_I according to the relationship $T_E = k T_I + m$ (k m constants

The *primary control of depth and rate of respiration* is accomplished through the rate of volume increase during the inspiratory phase In man at low drives with slow volume increases the operation is in range 1 where breathing rate is determined by some internal clock independent of volume As volume increases more rapidly operation moves into range 2 where both volume and rate increase with increasing ventilation In this volume dependent Hering Breuer range the larger volumes produce a shorter inspiratory duration according to the relationship (V_I V_0) $T_I = C$ (V_0 C constants Cats showed a pronounced range 2 with range 1 unobserved unless the vagi were cut After vagotomy range 2 operation was abolished and range 1 behaviour predominated as appears to be the case in man after vagal block (*Guz et al* 1966) If brief inflations were superimposed upon the normal breath during inspiration the inspiratory phase could be shortened but always according to the hyperbolic V_I—T_I relationship (Fig 1 The shape of the Hering Breuer range" was found not to be due to receptor properties but to the central organization

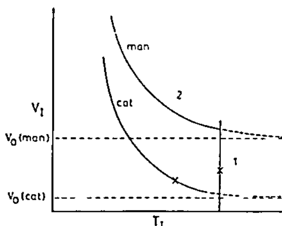


Fig 1 Diagrammatic representation of inspiratory volume (V_I) and inspiratory duration (T_I) for man and cat The curves are based on regression of experimental data and the V_I scales are normalized with respect to body weight In range 1 (1), T_I remains constant as V_I increases in response to increasing drive or imposed inflations In range 2 (2), T_I decreases with increasing V_I according to $(V_I - V_0)\ T_I = \text{const}$ The crosses indicate typical V_I—T_I values in eupnoic breathing

Inflations applied during the expiratory phase could prolong that phase These relationships proved to be independent of drive

References

GUZ A, M I M NOBLE J G WIDDICOMBE D TRENCHARD and W W MUSHIN *Respiration Physiol* 1966 *1* 206—210

C 3

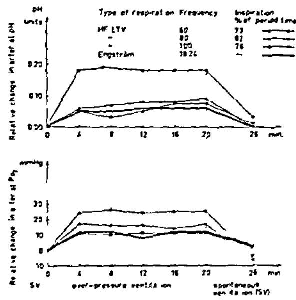
High Frequency Low Tidal Volume Positive Pressure Ventilation

By A JONZON P Å ÖBERG G SEDIN, U SJOSTRAND *Institute of Physiology and Medical Biophysics Biomedical Centre University of Uppsala, Sweden*

In order to eliminate the respiration synchronous arterial blood pressure variations in animal studies on the blood pressure regulation high frequency low tidal volume ventilation (HF LTV) was used In view of the usefulness of HF-LTV in animal experiments and its potential clinical applicability a study was made of its effects on the respiratory and circulatory physiology

Through a catheter inserted into the endotracheal tube atmospheric air was insufflated 60—100 times per min at excess pressure By means of a valve the expiratory flow and expiratory resistance could be regulated and thereby also the degree of pulmonary expansion When HF-LTV was started on dogs anesthetized with chloralose or Nembutal the spontaneous ventilation ceased instantaneously (possibly due to reflectoric mechanisms)

The intratracheal and intrapleural pressures and the arterial and central venous pressure (CVP) were recorded via transducers pO_2 pH and base excess in arterial blood were measured Expiration volumes of 5—20 l/min at insufflation frequencies of 60—100/min with varying relative insufflation times (20—86 % of the period time) were used and compared with conventional positive pressure ventilation wit an Engstrom respirator (ER)



Relative changes in pH and pO_2 with three settings of HF LTV and positive pressure ventilation with an Engstrom respirator (comparative study 7 dogs). Variations in pH and pO_2 in absolute values 7.30—7.48 and 80—110 mm Hg respectively

HF-LTV gave a mean variation in CVP of 10 mm Hg. The corresponding value with positive pressure ventilation with ER was 15 mm Hg. HF-LTV gave maximal intratracheal pressures of 8—12 cm H_2O—higher maximal pressures were recorded with ER (the mean pressures per time unit were not significantly different). Even during the insufflation phase HF LTV gave negative intrapleural pressures, which was not the case with ventilation by ER.

Measurements of the pulmonary perfusion (^{133}Xe) showed similar conditions for HF-LTV and ER ventilation. Nine hours of HF LTV gave no significant changes in base excess or compliance. Surviving dogs exhibited no respiratory difficulties or mental changes during an observation time of 1 week.

To summarize, the high frequency respiration described gives adequate ventilation, no negative effects on the circulation, low maximal intratracheal and intrapleural pressures and no metabolic disturbances.

C 4

Increased Activity in Vagal Cardiac Afferents Correlated to the Appearance of Reflex Bradycardia during Severe Hemorrhage in Cats

B. Öberg and P. Thorén, *Department of Physiology, University of Göteborg, Sweden*

With rapid severe hemorrhage in cats a sudden reflex bradycardia often emerges resembling the vasovagal syncope in man and probably initiated from receptors located in the heart (Öberg and White 1970). In the present experiments attempts were made to define the type and location of these proposed triggering receptors

Anesthetized cats were bled from one of the femoral arteries Blood pressure and heart rate was continuously recorded as well as impulse activity from thin filaments of the left vagus nerve in the neck (or of the right cardiac nerve) The remainder of the left and the intact right vagus nerves served as pathways for the reflex heart rate responses

With hemorrhage there was usually an initial tachycardia which with continued blood withdrawal was suddenly reversed to a bradycardia This bradycardia was preceded by a prompt increase of impulse activity from a low spontaneous discharge rate of 4—6 imp/sec to 20—30 imp/sec in thin probably unmyelinated vagal afferents This activity showed no cardiac rhythm With retransfusion of part of the shed blood the impulse activity abruptly decreased and the bradycardia reversed to a tachycardia

A similar increase of impulse activity, associated with a bradycardia was produced by partial occlusion of the ascending aorta by intrapericardial injections of nicotine (40 μg) and by pinching the ventricles Asphyxiation of the animal produced no or only a slight increase of impulse activity

Atrial receptor afferents regularly showed a markedly reduced activity during hemorrhage

The present data thus suggest that the reflex bradycardia produced by severe hemorrhage might be triggered from nicotine sensitive ventricular receptors probably mechanoreceptors showing a low spontaneous activity with no cardiac rhythm and signalling through thin probably unmyelinated fibres These receptors thus show a close resemblance to those described by Sleight and Widdicombe (1965) It is proposed that the receptors are activated when the ventricles contract vigorously around an almost empty chamber and then elicits a protective reflex permitting improved diastolic filling and preventing squeezing of the myocardium

References

Öberg B and S White *Acta physiol scand* 1970 In press
Sleight P and J G Widdicombe *J Physiol* Lond 1965 *181* 235—258

C 5

Nervous Influence on the Pulmonary Blood Volume

By P Aarseth *Institute of Physiology University of Oslo Oslo*

The lungs contain 10—15 per cent of the total blood volume in the body It is not clear how the volume of this large vascular bed alters with different circulatory conditions In the present experiments an attempt has been made to evaluate the immediate reduction of pulmonary blood volume upon a standardized blood loss and the role played by the vascular innervation in this connection

In rats a mixture of tagged erythrocytes (^{51}Cr) and tagged plasma albumin (^{125}I was injected Rapid arrest of the circulation was achieved by immersing the

thetized animals in liquid nitrogen Total blood volume and tissue blood content were estimated from the radioactivity in blood and tissue samples

Three groups of animals were used The animals in one group (group I) served as control The animals in the two other groups (group II and III) were bled about 12 per cent of their total blood volume just before immersion in liquid nitrogen In the animals of group III, thoracotomy had been performed and the left vago sympathetic nerve trunk had been cut 4 days before bleeding

The lungs of the bled animals in group II contained considerably less blood than did the lungs of the non bled control animals in group I The calculated average reduction of pulmonary blood volume in group II animals amounted to about 25 per cent In the animals in group III the pulmonary blood volume was on the average reduced by 10—20 per cent When compared to the animals in group I and II the left lung in these animals contained relatively more blood than did the right lung

The degree of blood mobilization from the vascular bed of skeletal muscle was also evaluated in the three groups One would expect this mobilization to be greater in the group III than in the group II animals This was found to be the case In group II animals the mean blood content of hind limb muscles was reduced by 14 per cent as compared to the control group animals In group III animals blood content of these muscles was on the average reduced by 28 per cent

In the present experiments the pulmonary vascular bed has thus to a substantial degree been functioning as a blood depot Moreover the mobilization of blood from this vascular bed is apparently to a large extent dependent on an intact vascular innervation

C 6

Microneurographic Analysis of the Fibre Spectrum of Human Sensory Nerve Fascicles

By R G Hallin and H E Torebjork *Department of Clinical Neurophysiology University Hospital Uppsala Sweden*

The evoked compound potential (ECP in response to electrical stimuli applied to a fingerpulp or a digital nerve was recorded in the median nerve and the superficial branch of the radial nerve at the wrist using the microneurographic technique introduced by Vallbo and Hagbarth 1968

The compound potential contains groups of components (Fig 1 which are recruited successively at increasing stimulus strength so that components with short latency corresponding to conduction velocities of about 50 60 m/sec are excited when the electric shock is just perceived by the subject whereas late components corresponding to conduction velocities ranging from 1 5—0 5 m/sec are not recruited until a sensation of pricking pain is experienced at each stimulus Prolonged compression of the nerve distal to the recording site without obvious ischaemic side effects in the hand abolishes the first components of the response whereas the late

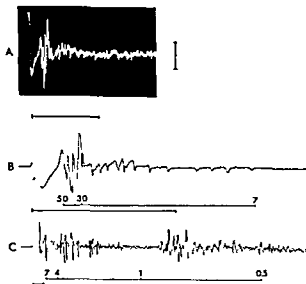


Fig 1 Electrically evoked compound potentials recorded from a median nerve fascicle at the wrist in response to short painful shocks in the hand The upward deflections indicate positive signals at the intraneural electrode Horizontal scales show the conduction velocity in m/sec for various components in the ECP Horizontal bars 10 msec in all recordings (A) A single ECP photographed from the oscilloscope screen Vertical bar 40 μV (B) 200 ECPs from the same recording averaged by a CAT computer (C) The same sequence as in B with a longer time base The averaged ECP contains late components reflecting activity in fibres with a conduction velocity as low as 0 5 m/sec

parts can still be recorded Furthermore the late parts of the ECP drop out before the first components when selectively blocking the nerve with 0 25 % Lidocaine The findings indicate that the first deflections of the ECP derive from activity in myelinated nerve fibres whereas the latest waves represent activity in C-fibres

Thus the microneurographic technique seems to be a feasible method to study impulse activity from both myelinated and unmyelinated nerve fibres in intact peripheral nerves in conscious man

References

VALLBO A B and K E HAGBARTH *Experimental Neurology* 1968 *21* 270—289

C 7

Excitatory and Inhibitory Propriospinal Pathways from Lumbo-Sacral to Cervical Segments in the Cat

By S MILLER *Department of Anatomy Rotterdam Medical Faculty Rotterdam The Netherlands*

Hemisection of the spinal cord at T_3 and L_1 results in marked degeneration ipsilaterally in the ventromedial part of the lateral motoneuronal cell group in C_7 T_1 (Sterling and Kuypers 1968 Giovanelli Barilari and Kuypers 1969) The degeneration occurs mainly among the motoneurones of the girdle muscles and largely avoids those of the arm muscles supplied by radial median and ulnar nerves (Fig 1 A) In acute spinal cats electrical stimulation of hindlimb nerves evokes a discharge ipsilaterally in pectoralis major and latissimus dorsi motoneurones but not in those of radial

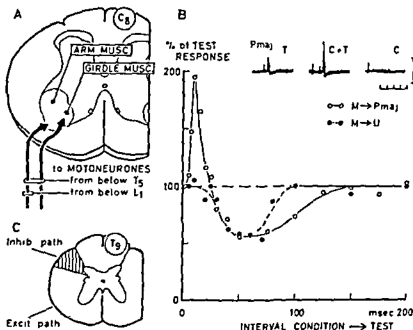


Fig 1 A Distribution of long ascending [illegible]
ment anatomical investigation (Sterlin[illegible]
1969) B Effects of hindlimb nerve c[illegible]
nerve to pectoralis major muscle (Pmaj) [illegible]
(M) to Pma[illegible] [illegible] shocks to ipsilateral quadriceps nerve at 5
x T M stimulated at 1 4 xT C Location of inhibitory and excitatory pathways at level of T_9
segment

ulnar (A weak excitatory effect is sometimes seen in median nerve see McDonald and McIntyre 1956) On reflex testing the hindlimb facilitation has a 5—12 msec onset and 10—25 msec duration In addition stimulation of both hindlimbs evokes inhibition of reflexes to all muscles mentioned above with 10—20 msec onset, 40 msec peak and 100 to 300 msec duration (Fig 1 B) In the T_9 segment the inhibitory path lies dorsally and the excitatory ventrally in the lateral funiculus (Fig 1 C) It is concluded that the excitatory path corresponds to the long ascending propriospinal projections demonstrated anatomically

References

GIOVANELLI BARILARI M and H G J M KUYPERS *Brain Res* 1969 *14* 321 330
McDONALD W I and A K McINTYRE *Proc Univ Otago Med Sch* 1956 *34* 5—6
STERLING P and H G J M KUYPERS *Brain Res* 1968 *7* 419 443

C 8

Ethanol Electrical Stimulation, and Net Movements of Sodium and Potassium in Rat Brain Tissue in Vitro

By P NIKANDER and H WALLGREN *Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki Finland*

Electrical stimulation of incubated cerebral cortex slices causes loss of intracellular potassium and gain of sodium Ethanol interferes with the generation of action potentials in single fibres acting predominantly on the rise in sodium conductance (Moore 1966) It has also been reported that ethanol inhibits reaccumulation of potassium in potassium depleted cerebral tissue (Israel Jacard and Kalant 1965) The aim of the present experiments was to differentiate between effects of ethanol on the response to electrical stimulation and the recovery after stimulation in terms of net ion movements

During a 10-min period of electrical stimulation ethanol (0 5 g/100 ml) strongly inhibited the rise in intracellular sodium with almost no effect on potassium loss This result is a parallel to the observation on single fibres in showing a selective action on sodium intrusion During recovery after 10-minutes stimulation, net extrusion of sodium is enhanced by ethanol whereas restoration of potassium is partially inhibited This result is not compatible with a generalized inhibition of active ion transport The observation suggests that structures sensitive to ethanol action may be situated on the outer surface of the excitable membranes

References

MOORE J W *Psychosom Med* 1966 28 450—457
ISRAEL JACARD Y and H KALANT *J cell comp Physiol* 1965 65 127—132

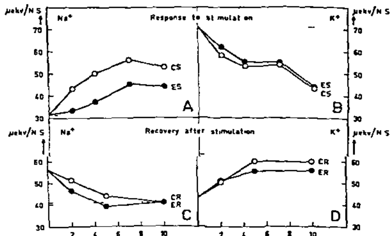


Fig 1 The effect of ethanol on net ion movements response to stimulation and recovery after stimulation The influx A and D is inhibited but not the efflux B) and C) The broken represents controls without stimulation CS control stimulation ES – ethanol stimulation control recovery ER ethanol recovery NIS = non inulin space

C 9

Proportionality between g-Strophanthin Binding Capacity and ATPase Activity

By O HANSEN *Institute of Physiology, University of Aarhus Denmark*

G strophanthin (ouabain) is known to be a specific inhibitor of the sodium pump of the cell and of the (Na —K) activated ATPase activity of cell membrane fragments Binding of labelled g strophanthin to the microsome fraction from ox brain was studied with a filtration technique where the parameter measured was the content of free g strophanthin of the filtrate The difference between added and free g strophanthin was taken to represent amount of g strophanthin bound to the membrane fragments The method allows a kinetic analysis of enzyme strophanthin interaction and thus an accurate estimation of maximum binding of g strophanthin.

Binding of labelled g strophanthin to the microsome fraction was shown to depend on the ionic milieu of the incubation medium With 5 mM Mg^{2} 2 mM EDTA 10 mM Na 3 mM ATP and 60 mM Tris (pH 7 4 37° C) nearly optimal conditions for g strophanthin binding were achieved i e a stable binding level was reached during a reasonable incubation time of maximally 45—60 min The absolute amount of g strophanthin bound (EG) at equilibrium varied with the concentration of free g strophanthin (G) but a saturation level was obtained with increasing g strophanthin concentrations Kinetic analysis of the equilibrium data for EG and G indicated that the binding process might be described simply by the model $E + G \underset{k}{\overset{k}{\rightleftharpoons}} EG$ An apparent dissociation constant (K_{app}^{EG} = 25 mμM and a value for EG_{max} was calculated based on the model presented If EG_{max} was plotted versus the specific total or (Na —K activated ATPase activity for enzyme preparations with activities varying from 30—272 resp 17—246 μmoles Pi released/mg protein/h a linear relationship between EG_{max} and ATPase activity was obtained Due to the small difference between the two ATPase activities of the single preparation it was not possible to decide if EG_{max} was better related to Na —K activity than to the total activity

C 10

Studies on the Action of Various Cyclic Nucleotides and their Metabolites on the Synthesis of Corticosterone in Vitro

By P BIECK, H VAPAATALO* and E WESTERMANN *Department of Pharmacology Medical School of Hannover Germany*

Cyclic adenosine 3' 5' mono phosphate 3 5 AMP has been suggested as a "Second Messenger" mediating many of the effects of a variety of hormones Sutherland et al 1968) it also takes part in the synthesis of corticosterone (Haynes et al 1959 Grahame Smith et al 1967 However the existence of other cyclic nucleotides acting as second messengers has been discussed Recently 3 5 GMP has been found in various

* Fellowship from the Alexander von Humboldt Stiftung

TABLE I Apparent affinities (K_A = moles liter) and intrinsic activities (E_{max} n moles corticosterone 100 mg tissue × hr) according to the terminology of Ariens *et al* (1956 were obtained from dose response curves which were plotted double reciprocally after Lineweaver and Burk

	K_A	E_{max}
ACTH		
3',5' AMP	3 5 × 10 [illegible]	101 4
N2 O-dibutyryl 3',5' AMP	3 0 × 10 [illegible]	110 2
3' 5' IMP	6 4 × 10 [illegible]	185,9
2' O-butyryl 3' 5' IMP	1 9 × 10 [illegible]	81 9
3' 5' UMP	6 9 × 10 [illegible]	98 1
2' O-butyryl 3' 5' UMP	~ 1 × 10 [illegible]	5
3' 5' GMP	2 9 × 10 [illegible]	52 1
	1 0 × 10 [illegible]	37 2

tissues and Glinsman *et al* (1969) have shown that its actions resemble those of 3',5' AMP

In the present paper it was studied, whether other cyclic nucleotides and their metabolites are able to affect the synthesis of corticosterone in rat adrenal slices (for details of the method see Bieck *et al* 1969) The potency of various nucleotides and their respective butyryl derivatives to stimulate corticosterone synthesis is shown in Table I

Without exception the more lipid soluble acyl derivatives of the nucleotides proved to be active in lower concentrations (K_A) than the parent compounds While the maximal effects (E_{max}) of 3' 5' AMP and 3' 5' IMP were in the same range that of 3' 5' GMP was markedly lower and that of 3' 5' UMP only slightly above control values

In addition various nucleosides and purine derivatives were tested in concentrations of 10^{-7} to 10^{-3} It was found that 5' AMP 5' IMP 5' GMP adenosine adenine and uric acid slightly stimulated steroidogenesis while 5' UMP hypoxanthine and xanthine were ineffective and inosine even inhibited it

It is suggested that at least 3' 5' IMP and 3' 5' GMP are able to substitute for the second messenger in corticosterone synthesis and that the accumulation of metabolites of the nucleotides may also affect steroidogenesis

References

ARIENS E J J M VAN ROSSUM and A M SIMONIS *Arzneimittel Forsch* 1956 *6* 282—293
BIECK P K STOCK and E WESTERMANN *Naunyn-Schmiedebergs Arch Pharmak exp Path* 1969 *263* 387—405
GLINSMAN W H E P HERN L G LINARELLI and R V FARESE *Endocrinology* 1969 *85* 711—719
GRAHAME SMITH D G R W BUTCHER R L NEY and E W SUTHERLAND *J Biol Chem* 1967 *242* 5535—5541
HAYNES R C S B KORITZ and F G PERON *J Biol Chem* 1959 *234* 1421—1423
SUTHERLAND E W G A ROBISON and R W BUTCHER *Circulation* 1968 *37* 279—306

C 11

Serum Non-Esterified Fatty Acids and Plasma Glycerol as Indicators of Fat Mobilization in Pregnant Sheep Subjected to Cold Stress

By N Astrup, A Allie, J F Nedkvitne and W Velle *Department of Physiology, Veterinary College of Norway, Oslo, and Institute of Animal Nutrition, the Agricultural College of Norway, Vollebekk, Norway*

The breeding season for sheep in Norway is in late fall, with lambing occurring in April/May The animals are normally kept in barns and shorn before the mating and again few weeks before parturition. The removal of the fleece increases their lower critical temperature from below zero to about 28° C (Blaxter 1962) Both the pregnant state and the high thermal conductance after shearing increase the maintainance requirements of the ewes In order to see to which degree the ewes mobilize their body fat to meet this increased requirements, the serum non-esterified fatty acids (NEFA) and the free plasma glycerol (PG) were followed during gestation in 15 ewes kept on a constant hay diet

During the two first months of gestation the body weight, NEFA and PG values varied little (NEFA 430 ± 175 μEq/l serum PG 39 ± 19 μmoles/l plasma) indicating adequate feeding During the following period NEFA and PG showed a parallel increase and reached maximal values at the first sampling after shearing (Fig 1 A) which coincided with a marked drop in the environmental temperature (Fig 1 B)

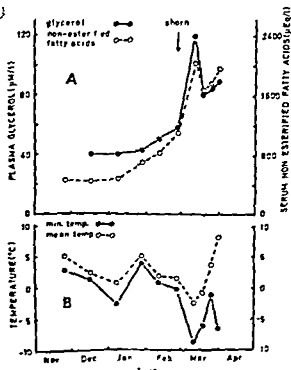


Fig 1
A The serum concentration of non-esterified fatty acids and the plasma concentration of free glycerol during the gestation period

B The mean and lowest temperature in the barn on the day of sampling

The metabolic rate after shearing was estimated to have been 2 to 4 times the basal rate This strong increase in the metabolic rate was not compensated by an increased food intake but resulted in a high degree of fat mobilization reflected by the marked rise in NEFA and PG during the last part of the gestation period The closely similar patterns of NEFA and PG indicate that both substances are good parameters for the degree of fat mobilization

References

BLAXTER (1962) *The energy metabolism of ruminants* Hutchinson and Co LTD (London)

C 12

Dynamics of Fluid Transfer between the Intra- and Extravascular Compartments during Exercise

By J LUNDVALL S MELLANDER H WESTLING and T WHITE *From the Department of Physiology and Biophysics and the Department of Clinical Physiology University of Lund*

Muscle activity leads to a pronounced transcapillary fluid movement from plasma into the extravascular space of exercising muscle due to increased hydrostatic capillary pressure (Kjellmer 1965) and to increased tissue osmolality (Mellander *et al* 1967) The oedema formation in exercising muscle was assessed both in cat and man from volumetric measurements of tissue mass after due correction for changes in regional blood volume Oedema formation was most rapid in initial stages of exercise and reached a steady maximal level within about 15 min When a small muscle group was exposed to heavy exercise (cat experiments) the cumulative fluid loss from plasma averaged 10 ml/100 g active tissue (*cf* Kjellmer 1964) During maximal exercise in man performed for 6 min on a bicycle ergometer the cumulative trans capillary fluid loss approached roughly 4 ml/100 g active tissue or a total of about 1000 ml assuming the active muscle mass to be 25 kg Since it is known that the net decrease of plasma volume is less than 500 ml in heavy exercise in man, compensatory mechanisms must be operating Our results suggest that maintenance of plasma vol ume at least in part is accomplished by absorption of extravascular fluid from resting tissues This appears to be caused both by reflex resetting of the pre to postcapillary resistance ratio and by osmotic factors The latter was suggested by the finding that arterial osmolality increased considerably (by 15—25 mOsm/kg H_2O) in both cat and man during heavy exercise engaging large muscle groups The hypothesis was tested in different ways Recording of transcapillary fluid shifts in the resting foreleg of the cat during exercise of the hindlegs showed an absorption of extravascular fluid averaging 1 5 ml/100 g resting tissue in 15 min About half of this effect could be ascribed to increased activity of the sympatho-adrenal system and the remainder to arterial hyperosmolality

Furthermore i v infusion of hypertonic solutions to resting man raising arterial osmolality by 10—15 mOsm/kg H O led to a true increase of plasma volume by 300

—400 ml and this effect was relatively well maintained for at least 30 min after cessation of infusion It appears that neurogenically induced absorption of extravascular fluid in exercise occurs mainly in skin and resting skeletal muscle, while osmotic absorption may be present in other resting tissues as well The arterial hyperosmolality in exercise may in addition, help to limit oedema formation in active muscle by reducing the transcapillary osmotic gradient between tissue and blood

References

KJELLMER I *Acta physiol scand* 1964 *62* 18—30
MELLANDER S B JOHANSSON S GRAY O JONSSON J LUNDVALL and B LJUNG *Angiologica* 1967 *4* 310—322

C 13

Increased Forearm Blood Flow during Ketoacidosis in Man

By S LEVIN NIELSEN *Institute of Medical Physiology, Dept A, University of Copenhagen Denmark*

Vascular resistance in the extremities in man during ketoacidosis developed under predominant fat metabolism has not been studied earlier although abnormalities in vascular reactivity are known to occur The purpose of the present study was to investigate the distribution pattern of the resting blood flow in extremities during ketoacidosis in man

Ketoacidosis was provoked in six young diabetic patients by reducing the insulin dosage and in five normal subjects by total starvation for 3 days Blood flow to both forearms was measured by venous occlusion plethysmography and blood flow to the cutaneous and subcutaneous tissue was determined by the local Xe^{133} clearance technique Muscle blood flow was calculated as the difference between these flow values and in addition determined in 6 of the subjects by the local Xe^{133} clearance method

In both diabetic and normal subjects blood flow in the resting forearm increased significantly during ketoacidosis (40 and 60 per cent respectively) due to augmentation in cutaneous and subcutaneous as well as in skeletal muscle blood flow The relative distribution of blood flow to the forearm tissues were approximately 1 1 2 (dermis subcutaneous tissue muscle both before and during ketoacidosis The increase in forearm blood flow was related to changes in standard bicarbonate glycerol and butyric acid but not to changes in blood pressure serum potassium or the calculated osmotic pressure The rest [illegible] time after 5 min of ischemia was prolonged in both groups during ketoacidosis

The cause of the vasodilatation seen in ketoacidosis is unknown In subcutaneous tissue vasodilatation is associated with [illegible]sed lipolysis

C 14

Physiological Interrelations during Intermittent and Continuous Exercise with the Same Average Power Output in Man

By R H T Edwards * C M Hesser, I Jack, A Melcher, A M McNinch R D W Wells, and O Wigertz *Department of Aviation and Naval Medicine, Karolinska Institutet and Clinical Physiology, Karolinska Sjukhuset, Stockholm, Sweden*

Three healthy male subjects performed, after an overnight fast 6000 kpm of work while seated on an electrically braked cycle ergometer Four paired comparisons between intermittent and continuous exercise with exactly the same average power output for the same total time, namely 250, 500, 800 and 1000 kpm/min, were made in each subject In the intermittent exercise a digital programmer controlled the ergometer load so that square wave work profiles of 10, 30 or 120 sec alternated with loadless pedalling recovery intervals of 30 sec duration Measurements made during exercise were separated from those during the whole recovery period of 30 min which included the 30 sec recovery intervals during intermittent exercise A reference control state of loadless pedalling at 60 rpm for 6 min prior to exercise was used when oxygen intake, heart rate, ventilation, and blood lactate concentration were respectively (mean ± SEM, n = 24) 464 ± 11 2 ml/min (STPD), 72 ± 2 2 beats/min, 13 2 ± 0 96 l/min (BTPS), and 0 76 ± 0 07 mM/l

Duplicate capillary blood samples for lactate estimation (based on Hohorst 1957) were taken at the end of the control and recovery periods respectively, and after completing 1/3, 2/3 and the whole of the work target Instantaneous heart rate and breath by breath ventilation were measured continuously (Wigertz 1970) and expired ventilation measured in four collections over the control first 2/3, and final 1/3 of the work target and the whole recovery period The total number of heart

Table I Results for Intermittent—Continuous exercise at all average power outputs

	Mean difference	SEM_{diff}	n	p	Mean % difference
Exercise Oxygen Intake (ml/min STPD)	76 5	19 5	24	<0 001	6 0
Exercise heart rate (beats min)	17 5	1 67	36	<0 001	18 0
Total Excess Heart Beats (beats)	222	62 8	11	<0 01	76 8
Exercise Ventilation (l/min BTPS)	7 4	1 19	24	<0 001	24 6
Exercise Lactate (mM/l)	0 7	0 12	36	<0 001	35 5
Perceived Exertion Grade (scale units)	2 1	0 38	12	<0 001	22 3

* In receipt of a Swedish Wellcome Travelling Research Fellowship

beats in these periods were summed with digital counters and the excess (work+recovery-control for same period) calculated Perceived exertion (Borg and Linderholm 1967) was graded immediately on completing the work target

Overall mechanical efficiency, the same for intermittent and continuous exercise (Christensen *et al* 1960), was 28 5 ± 1 0 (n = 21) per cent when related to the load less pedalling control in agreement with Whipp and Wasserman (1969) Respiratory exchange ratio and total excess ventilation were not significantly different, but exercise oxygen intake, ventilation heart rate, total excess heart beats blood lactate concentration and perceived exertion grade were higher with intermittent compared with continuous exercise (Table I) It was concluded that the performance of a given quantity of work in a set time is less stressful both subjectively and objectively when it is performed at a constant rate than when done intermittently

References

BORG G and H LINDERHOLM *Acta Med Scand* 1967 Suppl 472 194—206
CHRISTENSEN E H R HEDMAN and I HOLMDAHL, *Acta physiol scand* 1960 *48* 443—447
HOHORST H J *Biochem Z* 1957 *328* 509—521
WHIPP B J and K WASSERMAN *J Appl Physiol* 1969 *26* 644—648
WIGERTZ O *J Appl Physiol.* 1970 *29* 208—218

C 15

Hyperpolarizing Actions of Noradrenaline in Spinal Motoneurones

By I ENGBERG and A THALLER *Department of Physiology University of Goteborg Sweden*

In an attempt to analyse the action of noradrenaline (NA) on motoneurones Bruggencate and Engberg (1969) observed depression of discharges in some cells but no clear changes in membrane potential or conductance On the other hand Phillis *et al* (1968) reported hyperpolarizing actions by high doses of NA The present extension of the analysis suggests a mechanism that may explain the different results Repeated extracellular ejections of NA and control currents were performed on 50 intracellularly recorded motoneurones In 20 % of the cells a hyperpolarizing action of NA was demonstrated Fig 1 This effect was accompanied by a decrease in the amplitude of IPSPs and an inhibition of spike discharges An increase was observed in the amplitude of antidromically evoked spikes which was related to the amount of hyperpolarization. These NA effects seemed dependent on a high membrane potential they disappeared during the recording if the cells became depolarized but could occasionally be restored by a hyperpolarizing current injection NA was never found to evoke any membrane conductance increase This is in contrast to the very marked increase in conductance evoked by inhibitory amino acids — the NA hyperpolarization is apparently due to a different mechanism not involving an increased passive flux of ions (*cf* Libet and Kobayashi 1969

There is histological evidence for monoamine-containing nerve terminals close to motoneurones (Dahlstrom and Fuxe 1965 The present findings suggest that these

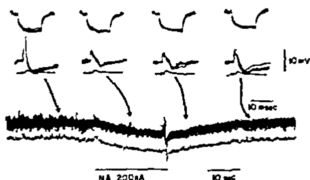


Fig 1 Intracellular records from motoneurone *Lower trace* Membrane potential record below which is indicated the duration of a microelectrophoretic noradrenaline (NA) application *Upper row of traces* Excerpted records of membrane potential changes produced by injection of short current pulses through the recording electrode indicating membrane conductance before, during and after the application of NA *Middle row of traces* Synaptic potentials and action potential evoked from the homonymous muscle nerve Note that the NA ejection hyperpolarizes the membrane and inhibits the action potential without any change in the membrane conductance

nerve terminals may exert inhibitory synaptic effects that differ in principle from the classical synaptic activity in the spinal cord Of particular interest in relation to this is the proposal by Siggins *et al* (1969) that NA stimulates cyclic adenosine monophosphate synthesis in cerebellar Purkinje cells Such metabolic changes may involve an active electrogenic mechanism responsible for the hyperpolarizing effect seen in the motoneurones

References

Bruggencate G and I Engberg, *Acta physiol scand* 1969 76 34 A—35 A
Dahlstrom A and K Fuxe *Acta physiol scand* 1965 *64* Suppl 247 7—85
Libet B and H Kobayashi *Science* 1969 *164* 1530—1532
Phillis J W A K Tebecis and D H York *European J Pharmacol* 1968 *4* 471—475
Siggins G R, B J Hoffer and F E Bloom *Science* 1969 *165* 1018—1020

C 16

Is Interruption of the Nigro-Striatal Dopamine System Producing the "Lateral Hypothalamus Syndrome"?

By U Ungerstedt *Department of Histology, Karolinska Institutet Stockholm, Sweden*

Since Anand and Brobeck (1951) found that bilateral lesions in the lateral hypothalamus caused aphagia there has been an increasing number of studies on this hypothalamic feeding center However some authors have questioned the concept of a centre and suggested that the lesion interrupts fibres passing through the area (Morgane 1961 The nigro-striatal dopamine (DA) system is one major pathway that ascends through the lateral hypothalamus (Anden *et al* 1966) but so far it has not been assigned any role in the lateral hypothalamic syndrome This study attempts to find out whether a lesion of the nigro-striatal DA system will produce aphagia and adipsia

It is known that an injection of 6-hydroxy-dopamine (6-OH DA) into the ca

after repeated doses of H44/68 was studied in the heart, innervated and decentralized submaxillary gland, seminal vesicle and vas deferens of the rat

A definite difference in effect of α-MPT on the different peripheral NA stores was demonstrated While the innervated hearts and submaxillary glands hat lost 22 and 36 % of their NA respectively in 10 hrs the short adrenergic neurons of the vas deferens and the seminal vesicle retained about normal NA levels even 16 hrs after repeated doses of α MPT, despite very high organ concentrations of the drug and regardless of whether the adrenergic nerve supply *via* the hypogastric nerve was intact or not It is concluded that this difference may be due either to a very low nerve impulse flow in the adrenergic nerves to the genital organs at rest or to a true difference in synthesis and storage mechanisms for NA in the short adrenergic neurons

References

CORRODI H and L ... 116—125
CORRODI H and T ...
NAGATSU T M LE ... —2917
SPECTOR S, A SJOE ... 65 147 86—95

C 19

Interference of Membrane Pump Blocking and α-Receptor Blocking Drugs with Noradrenaline Release from Isolated Tissues

By L-O FARNEBO and B HAMBERGER. *Department of Histology Karolinska Institutet Stockholm Sweden*

The effects of membrane pump blocking and α receptor blocking drugs on noradrenaline (NA) release and overflow from adrenergic nerves have been studied in several experimental models with divergent results (see *e g* Brown and Gillespie 1957 Thoenen *et al* 1964 a b Boullin *et al* 1967) Field stimulation of isolated tissue previously incubated with ^{3}H NA causes release of ^{3}H NA from the adrenergic nerves (Baldessarini and Kopin 1967 Farnebo and Hamberger 1970 Since vasomotor effects of stimulation presumably are avoided in superfused isolated tissues field stimulation was considered a useful technique for studies on the effects of drugs on NA release

Isolated irides and cerebral cortex slices prepared from untreated rats were incubated with ^{3}H NA 10 M in a Krebs Ringer bicarbonate buffer at 37 C After 30 min incubation the tissue was transferred to stimulation chambers through which buffer containing the drug to be tested was flowing After superfusion for 30 min the tissue was stimulated by an electrical field biphasic pulses 12 mA 2 msec 10 per sec) (Farnebo and Hamberger 1970 for 10 (iris or 2 (cortex min and then superfused for another 15 min The superfusate and the tissue (at the end of superfusion) were analyzed for total radioactivity

Field stimulation enhanced the overflow of tritium both from isolated irides and from cerebral cortex slices The stimulus induced overflow of tritium expressed as per cent of the total tritium content in the tissue at the onset of stimulation was 12

per cent for iris and 15 per cent for cerebral cortex The membrane pump blocking drug DMI caused a rather small increase of the overflow A larger increase was caused by the α-receptor blocking drugs phentolamine and phenoxybenzamine (10^{-6} M) In irides phenoxybenzamine 10^{-5} M a concentration which strongly inhibits the membrane pump, further increased the overflow The overflow with phentolamine or phenoxybenzamine 10^{-6} M could be augmented with DMI

It could be expected that DMI should cause a large increase of the overflow as a consequence of inhibited reuptake of NA No such large increase was obtained and it may thus be assumed that the actual release from the nerve terminals was decreased Blockade of the α receptors caused a marked increase of the overflow suggesting an increased release from the nerve terminals In agreement with Haggendal (1970) it seems as if the receptor organ is able to influence the amount of transmitter released from the nerve terminals

References

BALDESSARINI, R J and I J KOPIN J Pharmacol exp Ther 1967 156 31—38

BOULLIN D J, E COSTA and B B BRODIE, J Pharmacol exp Ther 1967 157 125—134

BROWN, G L and J S GILLESPIE J Physiol (Lond) 1957 138 81—102

FARNEBO L O and B HAMBERGER J Pharmacol exp Ther 1970 172 332—341

HAGGENDAL, J, In New aspects of storage and release mechanisms of catecholamines Bayer Symposium 1970 Springer Verlag Heidelberg In press

THOENEN, H, A HUERLIMANN and W HAEFELY J Pharmacol exp Ther 1964 a 143 57—63

THOENEN H, A HUERLIMANN and W HAEFELY Helv physiol pharmacol Acta 1964 b 22 148—161

C 20

Studies on Ribonucleic Acids of the Mammary Gland of Pregnant and Lactating Rabbits

By A DEUTSCH and A NORGREN Department of Biochemistry, Chemical Center and Institute of Physiology and Medical Physics University of Lund Sweden

Development of the mammary gland during pregnancy is accompanied by an increase in both RNA and DNA contents With the onset of secretion a rapid increase in RNA takes place both in relation to wet weight and DNA content the increase in RNA being most marked after parturition and declining rapidly after weaning (Denamur, 1964)

In order to assess changes in the RNA pattern related to changes in total RNA content between pregnancy and lactation RNA was isolated from the mammary gland of rabbits at mid pregnancy and at the height of lactation (15 days after parturition) The effect of engorgement of the gland with milk similar to that at weaning, was studied by using the technique of unilateral ligation of the teats of lactating rabbits

1 Our results show a disproportionate increase in ribosomal RNA in relation to total RNA between mid pregnancy and the height of lactation Similar results were obtained regardless of the method used for isolation of RNA (extraction with phenol

C 23

Changes in Spinal Excitability during an Auditory Vertex Response in Man

By H. Fruhstorfer,* T. Järvilehto,* J. Lumio, P. Soveri, *Institute of Physiology, University of Helsinki, Finland*

In man a sudden auditory stimulus is usually followed by a vertex response and a startle reaction. The component N_1 of the vertex response and the early components of the startle reaction appear approximately synchronously, they are symmetrical and dependent on the stimulus intensity, they habituate when the stimulus is repeated and they disappear in sleep (*cf.* Larsson 1956). Because of their similar characteristics it may well be that these phenomena are mediated by a common neural mechanism which has both central and peripheral excitatory influences. This hypothesis would be further supported if the time course of the changes in spinal excitability after an auditory stimulus were similar to the time course of the auditory vertex response.

Four subjects received click stimuli of medium intensity and after each stimulus at varying time intervals the excitability of the spinal motoneurons was tested by H reflexes. They were elicited by giving an electric shock to the n. tibialis posterior in the popliteal fossa and the EMG activity was recorded from the lateral edge of the soleus muscle.

The amplitude of the H reflex was significantly dependent on the interval between the auditory stimulus and the electric shock (one-way analysis of variance, $p < 0.01$). Individual comparisons revealed that the reflexes elicited 55—125 msec after the stimulus were significantly larger than the reflexes elicited with delays of 175—230 msec (confidence limits calculated by *q*-statistics, $p < 0.01$). These interval ranges correspond well to the durations of the components N_1 and P_2 of the auditory vertex potential.

The results support the hypothesis that the auditory vertex response and the excitation of spinal motoneurones as indicated by a startle reaction are mediated by the same functional system. It has been shown in cat that the auditory startle reaction involves a series of neurons in the nucleus gigantocellularis of the brain stem (Hossmann 1969) and that this region has also extensive connections to the centre median of the thalamus which projects to cortical association areas (Bowsher *et al.* 1968). In man a corresponding system of descending and ascending connections could form a common neural basis for the generation of the startle reaction and the vertex response.

References

Bowsher, D., A. Mallart, D. Petit and D. Albe-Fessard: *J. Neurophysiol.* 1968. 31. [illegible]—300.

Hossmann, K. A.: *Exp. Brain* [illegible] 43 [illegible]

Larsson, L. E.: *Elect.* [illegible] 1956. 8. 631—644.

* Present address: [illegible] Universität Marburg, D 355 Marburg/La., Germany.

C 24

Response Patterns of Human Muscle Spindle Endings to Isometric Voluntary Contractions

By A B VALLBO Dept of Physiology, Biological Institute University of Umeå Sweden

The skeletomotor as well as the fusimotor system may be activated from the cerebral motor cortex by electrical stimulation (Vedel and Mouillac Baudevin 1970) However, the effect of such stimulation is not uniform with regard to the relation between the skeletomotor activity and the spindle response the discharge rate from the receptor may rise without a simultaneous skeletomotor activity, the two of them may increase together, or the skeletomotor activity alone may increase under isometric conditions, as shown in the primate (Koeze 1968 Koeze Phillips and Sheridan 1968) Voluntary contractions in man are also associated with adjustments of the fusimotor activity (Hagbarth and Vallbo 1968 Hagbarth and Vallbo 1969 Vallbo 1970)

In the present study, activities from single spindle endings were analyzed in waking human subjects during isometric voluntary contractions with the purpose to find out if a uniform type of receptor response was induced in the intact organism under these conditions, or if not which were the factors determining the response patterns Impulses from single muscle spindle endings in the wrist and finger flexor muscles were recorded with percutaneously inserted tungsten electrodes from the median nerve

Three main types of response patterns were encountered a sustained increase of the impulse rate, a sustained decrease and a weak and variable discharge consisting of a few impulses A certain type of pattern was not characteristic of certain spindles but many spindles responded with different patterns It was found that the intensity and particularly the spatial extent of the skeletomotor contraction were significant factors for the response pattern of a certain spindle For the majority of the endings a sustained increase of the impulse rate could be demonstrated when the subject activated a certain portion of the muscle system Several findings indicated that the receptor was located in this muscle portion Contractions of neighbouring portions often induced either a weak discharge or a decrease of the discharge These effects might be accounted for by mechanical factors More remote contractions had no effect on the impulse rate

It was concluded that there was a close spatial correspondance in the muscle between the skeletomotor and the fusimotor activity

References

HAGBARTH K E and A B VALLBO Exptl Neurol 1968 22 674—694
HAGBARTH K E and A B VALLBO Acta physiol scand 1969 76 321—334
KOEZE T H J Physiol (Lond) 1968 197 87—105
KOEZE T H C G PHILLIPS and J D SHERIDAN J Physiol (Lond) 1968 195 419—449
VALLBO A B Acta physiol scand 1970 78 315—333
VEDEL J P and J MOUILLAC BAUDEVIN Exp Brain Res 1970 10 39—63

Thalamo Cortical Rhythmicity During Reversible Visual Deafferentation

By S. Andersson, E. Holmgren and T. Ivarsson *Department of Physiology, University of Göteborg, Sweden*

A reduced afferent discharge in specific spinal pathways facilitates the generation of rhythmic activity in the alpha frequency range in the thalamic somatosensory projection nuclei and the appropriate parts of the somatosensory cortex due to interferences with the rhythm generating mechanism at the thalamic level (Andersson, Holmgren and Manson 1971).

In this study the visual system was investigated with regard to thalamo cortical activity during deafferentation. The activity in the visual cortex and corpus geniculatum laterale (GL) was recorded in cats under light barbiturate anaesthesia or in unanaesthetized decorticate preparations. Recordings from other cortical and thalamic regions were often made simultaneously. The afferent visual inflow was reversibly blocked by an increase of the intraocular pressure to a suprasystolic level.

During light barbiturate anaesthesia the background activity showed similar periods of rhythmicity both in thalamus and in cortex. About one minute after the application of an increased intraocular pressure the flash induced focal potentials in GL and occipital cortex began to decrease and disappeared completely some half a minute later. At this time the rhythmicity was strongly enhanced with longlasting well defined periods of regular waves within the frequency range of 8—12/sec both in GL and in the occipital cortex. Frequently the rhythmicity became continuous. When the intraocular pressure was released after 3—5 minutes the amount of synchronous activity was reduced to the resting level with the return of the evoked responses.

In the unanaesthetized decorticate preparation the spontaneous activity was sometimes completely desynchronized with many cells firing at a high frequency. However during the periods of visual deafferentation the background activity in GL decreased markedly and longlasting spindles or a continuous rhythmicity appeared while the change in neighbouring parts of thalamus was only slight if any. The change in activity during and after the periods of increased intraocular pressure was closely related to the disappearance and return of the response evoked by a flash. No clear change in thalamic activity occurred if the evoked response was not completely eliminated.

The findings indicate that components of the specific projection systems have important effects upon the rhythmgenerating mechanism in the thalamic relay nuclei. It is suggested that impulses in specific afferent fibres have a desynchronizing action via excitation of the relay cells and inhibition of the inhibitory interneurone in the recurrent loop.

References

Andersson, S. A., E. Holmgren and J. R. Manson, *Electroenceph. c*
In press

C 26

ıfluence of Plasma Hypertonicity on Blood Viscosity Studied in Vitro and in an olated Vascular Bed

By S NYBO RASMUSSEN *Institute of Medical Physiology B University of Copenhagen Denmark*

ıcreased viscosities of blood with increasing osmolality have been demonstrated in *tro* after addition of nonpermeant solutes *e g* NaCl or mannitol (Rand and ıcombe 1964 and others) Schmid Schonbein and Wells (1969) demonstrated that low shear rates the viscosity of blood with osmotically crenated red cells is reduced ˈcause of abolished aggregation whereas at high shear rates the viscosity is in eased due to reduced viscous deformability (fluidity) of the shrunken cells

The purpose of the present study was to examine the effects of plasma hyper nicity on blood viscosity in an isolated vascular bed as well as in pure *in vitro* periments NaCl was added to oxblood to produce hypertonicity The numbers of d cells in isotonic and hypertonic blood from the same batch were kept identical he isolated rabbit ear was perfused at 70 and 30 mm Hg Alterations in vascular sistance were abolished by adding a vasodilator drug to all perfusion media The pparent viscosities of the red cell suspensions relative to the suspension media were ılculated from the corresponding flow ratios Relative *in vitro* viscosities of the ıme samples were determined by means of a cone plate viscometer at definite shear ıtes The experiments were divided in four groups according to the level of hyper onicity The mean values for the hypertonic samples were 415 535 735 and 950 ıosm/kg Both the relative viscosities measured *in vitro* and *in vivo* were increasing ıth increasing osmolality the maximal increments amounting to about 100 per cent ˈhe increase in viscosity was closely correlated to the MCHC The relative apparent ıscosities obtained at 70 mm Hg were significantly lower than those obtained *in vitro* t any shear rate The relative viscosities obtained at 30 mm Hg were close to those btained *in vitro* and were sometimes greater than these

It is concluded that hypertonicity greatly influences the viscosity of whole blood *n vivo* as well as *in vitro* This effect must be attributed to the red cells and it may ıave important consequences in the renal medullary circulation The inverse rela ıonship between renal medullary blood flow and the medullary hypertonicity lemonstrated by Thurau *et al* (1960) and others may at least in part be explained ɔy changes in viscosity of the blood during its passage through the vasa recta system

References

RAND P W and E LACOMBE *J Clin Invest* 1964 *43* 2214—2226
SCHMID-SCHONBEIN H and R WELLS *Pflugers Arch* 1969 *307* 59—69
THURAU K P DEETJEN and K KRAMER *Pflugers Arch* 1960 *270* 270—285

C 27

Effl ıx of Prostaglandıns ın Lymph from Scalded Tissues

By E ÄNGGÅRD G ARTURSON and C E JONSSON *Burn Center, Uni ersit Hospital, Uppsala and Department of Pharmacology, Karolinska Institutet Stockholm Sweden*

Sev ral endogenous mediators have been suggested to participate in the inflam mator reaction following a burn injury Of these histamine is connected with th early phase of vasodilation and increased capillary permeability As mediators fo the l ite phase kinins and various permeability factors have been suggested (Spect r and Willoughby 1968) Willis (1969) have described prostaglandin like activity ir carrageenin induced inflammation in the rat

Sm oth muscle stimulating activity was found in paw lymph of the dog after bu not l efore scalding (Jonsson to be published) The spasmogenic activity was un affected by antagonists for acetylcholine, histamine and serotonin and was not in activated after incubati n with chymotrypsin which excludes the possibility of poly peptides On isolated g inea pig ileum the activity was typically 'slow reacting' In preliminary experiments it was found that a great deal of the biological activity could be recovered in a lipid fraction of lymph This indicated that prostaglandins were present and work was initiated to identify the activity (Änggård and Jonsson, to be published)

Almost all smoo th muscle contracting activity (rat fundus strip) in an alcoh lic extract of lymph was recovered in the acidic lipid fraction This fraction was co chromatographed with PGE ^{3}H on silicic acid for group separati n of prosta glandins and on hydrophobic kiselguhr in a reversed phase partition system f r separation of individual PGE-compounds Fractions were assayed for bi logical ac tivity and radi activity In both systems these two usually coincided

When biol gical activity from silicic acid chromatography was incubated with 15-hydroxy pr staglandin dehydrogenase from swine lung and NAD complete in activation was achieved

Active material rec vered from silicic acid and reversed phase partition chroma tography was tre ated with alkali and methylated The material was put on a lipo philic Sephadex c lumn The methylester was isolated and treated with silyl re agent and examined by GLC with an electron capture detector The lymph ma erial gave an EC-response with identi al retention time as the same derivative of authe ic PGF-

The results sh w that PGF is f und in paw lymph following a scald ng inj ry of the dog Prelimin ary results sh w that human burnblister contains smooth m scle stimulating activity in the acidic lip id fraction

The potent effects f pr staglandins on vessels (tonus and permeab lity) kidney and lipid metabolism Bergstr m *et al* 1968) make this finding interesting Perhaps some of the local or system ic m anif tation s of the burn syndrom can be explained by the presence of pro aglan d s

References

Bergstrom S L A Carlson and J R Weeks *Pharmacol Rev* 1968 *20* 1—48
Spector W G and Willoughby The pharmacology of inflammation The English Universities Press Ltd, London 1968
Willis A L *J Pharm Pharmac* 1969 *21* 126—128

C 28

The Permeability of the Blood-Brain Barrier to Lactate and Pyruvate

By C Crone and S C Sorensen *Institute of Medical Physiol Dept A & B, University of Copenhagen Denmark*

The brain continuously produces lactate (Cohen *et al* 1967, Sorensen *et al* 1969) During hypoxia and hypocarbia anaerobic glycolysis increases Under steady state conditions production of lactate must equal removal by the blood In general even smaller crystalloids and ions pass the blood brain barrier slowly There are exceptions to this rule, the most prominent being D glucose, which passes the blood brain barrier easily due to a carrier mediated facilitation of glucose diffusion

The concentration difference for lactate between brain extracellular fluid and blood is presumably small and one might therefore expect that the transport of lactate from brain into blood also takes place by a process of facilitated diffusion

It is difficult to assess the permeability of the blood brain barrier to lactate when it passes from brain into blood By means of the single injection technique (Crone 1963 the transport rate in the opposite direction can be determined If the system is symmetrical — as in simple or facilitated diffusion processes — the transport of lactate from blood into brain should be as great as that from brain into blood.

Experiments were carried out in dogs which received C^{14} labelled D and L-lactate in single carotid arterial injections together with a non permeable reference tracer H^3 mannitol Immediately after the injection the effluent blood from the superior sagittal sinus was collected analyzed and compared with the composition of the injectate

In five experiments there was no measurable extraction of either D lactate or L-lactate within the limits of sensitivity of the method

We considered that pyruvate might be the form in which the products of anaerobic glycolysis are removed from the brain However in five experiments C^{14} pyruvate displayed the same low permeability as that found for lactate

Lactate and pyruvate thus conform to the general pattern of most polar substances that of an impeded exchange between blood and brain tissue

References

Cohen P J S C Alexander T C Smith M Reivich and H Wollman *J appl Physiol.* 1967 *23* 183—189
Crone C *Acta physiol scand* 1963 *58* 292—305
Sorensen S C J S Milledge and J W Severinghaus *Fed Proc* 1969 *28* 337

C 27

Eff[illegible]ts of Prostaglandins in Lymph from Scalded Tissues

By E Änggård G Arturson and C E Jonsson *Burn Center Unive Hospital, Uppsala and Department of Pharmacology, Karolinska Instit Stockholm, Sweden*

Sev ral endogenous mediators have been suggested to participate in the inf matory reaction following a burn injury Of these histamine is connected with early phase of vasodilation and increased capillary permeability As mediator the late phase kinins and various permeability factors have been suggested (Sp and Willoughby 1968) Willis (1969) have described prostaglandin like activi carrageenin induced inflammation in the rat

Smooth muscle stimulating activity was found in paw lymph of the dog after not before scalding (Jonsson to be published) The spasmogenic activity was affected by antagonists for acetylcholine histamine and serotonin and was not activated after incubation with chymotrypsin which excludes the possibility of p peptides On isolated guinea pig ileum the activity was typically slow reacting preliminary experiments it was found that a great deal of the biological act could be recovered in a lipid fraction of lymph This indicated that prostaglan were present and work was initiated to identify the activity (Änggård and Jon to be published)

Almost all smooth muscle contracting activity (rat fundus strip) in an alcoh extract of lymph was recovered in the acidic lipid fraction This fraction was chromatographed with PGE ^3H on silicic acid for group separation of pro glandins and on hydrophobic kiselguhr in a reversed phase partition system separation of individual PGE-compounds Fractions were assayed for bi logical tivity and radioactivity In both systems these two usually coincided

When biological activity from silicic acid chromatography was incubated w 15-hydroxy prostaglandin dehydrogenase from swine lung and NAD complete activation was achieved

Active material recovered from silicic acid and reversed phase partition chr tography was treated with alkali and methylated The material was put on a l philic Sephadex column The methylester was isolated and treated with chlat agent and examined by GLC with an electron capture detector The lymph ma gave an EC-respon e with identic l retention time as the same derivative of authen PGE.

The results show that PGE is f und in paw lymph following a scalding injur the dog Preliminary results show that human burnblister contains smooth m stimulating activity in the acidic lip d fraction

The potent effect f pr staglandins on vessels (tonus and permeability) lid and lipid metabolism Bergstr m *et al* 1968) make this finding interesting Perh some of the local or system c m nifestations of the burn syndrom can be expla by the presence f prostaglandins